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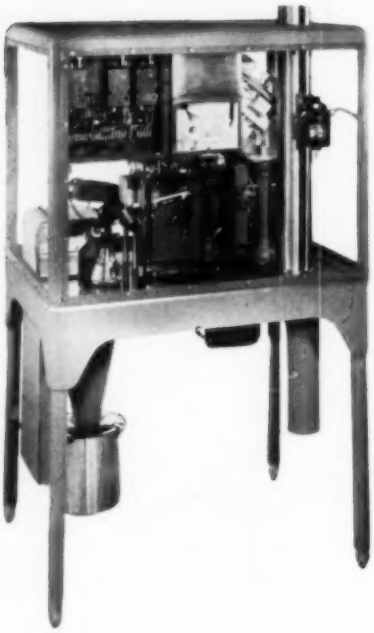

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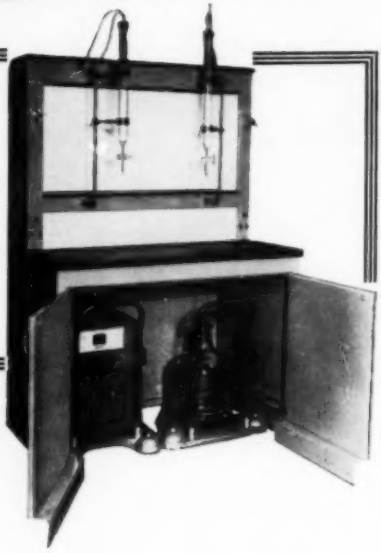

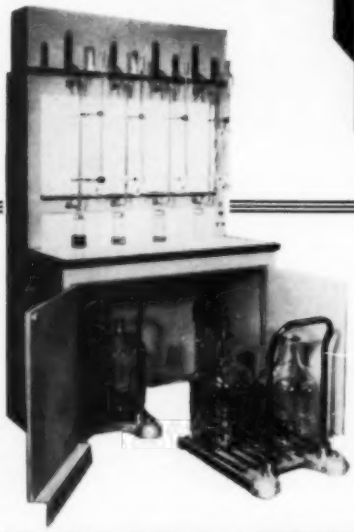

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CEREAL CHEMISTRY

VOL. 36 JULY, 1959 NO. 4

STUDIES ON THE PHYSICAL NATURE OF GLIADIN[1]

JOHN HOLME[2] AND D. R. BRIGGS

ABSTRACT

Crude gliadin, as prepared by the extraction of gluten with 0.07*M* acetic acid or with 70% aqueous ethanol, contains, to the extent of about 90% of the mass of the extracting material, a component which appears homogeneous in the ultracentrifuge. This component, which may be designated as true gliadin and is obtained in small yield through fractional precipitation of a solution of crude gliadin in 60% ethanol, has a molecular weight of about 25,000 as determined by ultracentrifuge or by light-scattering methods.

Despite a marked hydration capacity, gliadin at neutral reaction exhibits a strong aggregation tendency through intermolecular hydrogen bonds. While water cannot successfully compete with this intermolecular cross-bonding to carry the gliadin into molecular solution, the imposition of a small charge on the protein, in absence of other electrolytes, or the addition of urea in an amount to yield a 1- to 2-molar solution, will do so. Under these conditions, the molecular kinetic properties of gliadin can be studied. Evidence is presented that the sites of intermolecular cross-linkage involve the amide groups of the protein.

The chemical composition and physical behavior of gluten proteins have long been subjects of interest to protein chemists and of great practical importance to the baking industry. The results of numerous fractionation techniques have, in great part, led to a belief that the material is a complex mixture of a number of different protein components. The work of Cook (11), Sandstedt and Blish (25), and McCalla and co-workers (21,22,28) has indicated that gluten can be made to yield a multitude of fractions which differ progressively and systematically in both chemical and physical properties, depending on the method of separation.

The cohesive, doughlike nature of the hydrated gluten mass is retained in hydrated gliadin, the prolamine of wheat flour, first reported by Taddey (2) and commonly prepared by the extraction of gluten

[1] Manuscript received October 1, 1958. Contribution from the Department of Agricultural Biochemistry, University of Minnesota, St. Paul, Minnesota. Paper No. 3986, Scientific Journal Series, Minnesota Experiment Station. A part of a thesis submitted to the Graduate School of the University of Minnesota by J. Holme in partial fulfillment of the requirements of the Ph.D. degree. This study was aided by a fellowship donated by the Procter & Gamble Co.

[2] Present address, Procter & Gamble Co., Cincinnati, Ohio.

with 60–70% aqueous ethanol (14) or 0.07*N* acetic acid (5). The occurrence of these dough-forming properties in this fraction of the total gluten complex presents the possibility that a better understanding of the function of gluten in dough behavior can be gained through a physicochemical study of gliadin.

The results of many studies on gliadin in the past have failed to reveal an explanation of the physical peculiarities of the substance, but the recent work by Cunningham *et al.* (12) has suggested a correlation of certain elements of the chemical composition of the protein with its physical properties. Evidence for heterogeneity of gliadin has been indicated by such methods of investigation as optical rotation and viscosity (17), ultracentrifugal sedimentation (18), diffusion (19), osmotic pressure (6), and electrophoresis (10,23,26).

A characteristic property of gliadin is its low buffer capacity through a range of 2 to 3 pH units on either side of pH 7. In this pH range, gliadin, in contact with aqueous solvents, forms a cohesive, pliable, hydrated mass but shows virtually no actual solubility in the solvent phase. At pH values below 4, however, the dispersibility of the salt-free protein in salt-free water increases rapidly with decreasing pH but is extremely sensitive to a small increase in ionic strength. Whereas titration of a gliadin suspension with hydrochloric acid to pH 3.5 will result in a nearly water-clear solution at a protein concentration of 5% or greater, the presence of salt (sodium chloride) at 0.05 ionic strength will prevent dispersion as completely as would be the case at pH 7. There obviously exists a marked association tendency between the gliadin molecules which prevents their passage into molecular dispersion in water in spite of a distinct hydration capacity. The imposition of a small charge upon the gliadin molecules apparently shifts the balance of forces toward an increased attraction for water to such an extent as to allow the protein molecules to pass into solution. Increase in ionic strength lowers the effectiveness of the imposed charge and reverses the dispersion tendency in favor of the intermolecular attractions existing between the protein molecules.

Schwert, Putnam, and Briggs (26), in their studies on the electrophoretic properties of gliadin, employed the conditions of pH 3.8 and ionic strength of 0.01. Under these conditions and at 1% concentration, gliadin yields a nearly water-clear solution. The electrophoresis patterns obtained invariably showed a gross nonenantiography between the ascending and descending boundaries. These authors reasoned that the protein is electrophoretically nonhomogeneous and that the components, even under this condition of apparent molecular solution, still show a residual amount of the interaction tendency

exhibited in the wet-bulk phase. Smith and Briggs (27) demonstrated, using bovine serum albumin and methyl orange systems, that interaction of these components in solution results in nonenantiography of electrophoretic patterns obtained on such systems, thus giving support to the interpretation proposed for the gliadin patterns.

Experiments have been made which were aimed at testing this interaction hypothesis of the source of the observed anomalous electrophoretic patterns of gliadin and at ascertaining the functional groups responsible for such intermolecular association in the protein. The types of interaction forces which can be postulated as operating between the protein molecules in solution include hydrogen bonding, electrostatic bonding, and dispersion or Van der Waals' association forces. Solvent compositions which might be expected to overcome one or more of these interaction forces were employed in studying the electrophoretic behavior and the particle size properties of gliadin in solution. The effect of chemical modification of the functional group composition of the protein upon certain of these physical properties has been investigated. Finally, fractionation techniques have been employed to obtain information regarding the particle size homogeneity of gliadin preparations.

Materials and Methods

Electrophoresis. Electrophoretic analyses were performed at 4°C. with the Klett-Tiselius apparatus equipped with a cell as modified by Alberty (1). The Philpot-Svensson cylindrical lens system was used to follow pattern development, and photographic records were obtained either by this system or with the Longsworth scanning technique. All solutions were equilibrated against a suitable volume of buffer by dialysis on a rotating dialyzer at 4°C. Mobilities and areas were obtained from enlarged traces of patterns projected onto cross-sectional paper.

Ultracentrifugation. Sedimentation studies were made at room temperature with a Model E Spinco analytical ultracentrifuge. Measurement of distances moved by boundaries was carried out in a way similar to that used for electrophoresis. Sedimentation values were corrected to standard conditions (water at 20°C.) by the method of Svedberg and Pedersen (29). Viscosity values of urea-buffer solutions were obtained at 18.7° and 24.1°C. with an Ostwald-Fenske viscometer described by Cannon and Fenske (9). These values were joined graphically by a straight line over the temperature range encountered, and values for the viscosity at any intermediate temperature were obtained by interpolation. The density of this buffer solution was determined

pycnometrically at 21.6°, 23.1°, and 26.5°C. Values at any temperature encountered in sedimentation were obtained from a graph joining these three values.

Light Scattering. Turbidity measurements were made at 436 $m\mu$ with a light-scattering photometer built in this laboratory by Bleidner (4) and used for protein study by Fuller and Briggs (16). All solutions were pressure-filtered through ultrafine sintered glass filters. Refractive index increments were determined at 436 $m\mu$ with a Phoenix differential refractometer. The turbidities of solutions, measured at 90° to the incident beam, were determined relative to a benzene standard (τ Std. $= 8.0 \times 10^{-4}$ (3)) at 436 $m\mu$. Weight average molecular weights were determined from the intercept of plots of Hc/τ against concentration extrapolated to $c = 0$, and in some cases corrected for depolarization (13). The constant H, from the general equation for molecular weight $Hc/\tau = (1/M) + 2Bc$, was calculated from

$$H = \frac{32\pi^3}{3} \frac{n_0^2}{\lambda^4 N} \left(\frac{n - n_0}{c} \right)^2$$

where
- c = concentration in g. per cc.
- N = Avogadro's number
- n = refractive index of solution
- n_0 = refractive index of solvent
- λ = wave length of primary radiation *in vacuo*

Gliadin. Various preparations of crude gliadin were employed in this study. These were obtained by the 70% ethanol extraction method of Dill and Alsberg (14) or the acetic acid method of Blish and Sandstedt (5). No significant distinction was observed between the crude gliadins obtained by the two methods.

Hydrolysis of Primary Amide Groups of Gliadin. A method very similar to that of Vickery (31) was employed to effect partial deamidation of gliadin. Hydrolysis was brought about with hydrochloric acid at concentrations ranging from 0.008 to 0.04*N* at the temperature of a boiling-water bath. The extent of amide hydrolysis was determined by collection and titration of the ammonia contained in an aliquot of the hydrolysis mixture. Following hydrolysis, the solution was neutralized, and dialysis was employed to remove salts prior to lyophilization. In some cases, the aqueous protein solution was dialyzed directly against buffer suitable for electrophoresis.

Low-Temperature Alcohol Fractionation of Gliadin. A thermal fractionation of gliadin was carried out according to the method of Haugaard and Johnson (17). Thirty grams of gliadin were mixed with 300 ml. of 60% aqueous ethanol for 24 hours at room temperature.

The gravitationally stable suspension was kept at 0°C. for several days, after which time a firm gellike residue (about 50% of the original protein) was collected. This was designated as fraction F_1. The clear supernatant from F_1 was held at −10°C. for several days and yielded a residue, F_2 (25% of the original protein). The turbid overlying solution was coagulated by treatment with absolute ethanol to yield a fraction, F_3, representing 18% of the original gliadin. A portion of F_3 was returned to 60% aqueous ethanol at a 10% concentration, and this solution was cooled for several days at −10°C. Part of the dissolved material precipitated. The turbid supernatant was collected, and the protein was recovered by precipitation with ethanol. This was called F_4 and constituted about 8% of the original gliadin. Fractions F_2, F_3, and F_4 were dehydrated and powdered in absolute ethanol and ether prior to being redissolved in the standard solutions employed in light-scattering and ultracentrifuge examinations.

Bovine Plasma Albumin. The sample was an Armour crystallized preparation (lot No. N67210).

Ovalbumin. The sample was kindly supplied by R. A. Fuller of this department, and was prepared according to the method of Larson (20).

Casein. The sample was kindly supplied by R. Jenness of this department and was prepared by the Van Slyke-Baker method (30); it contained 0.832% phosphorus and 15.93% nitrogen.

Polymethacrylamide. Methacrylamide (kindly supplied by the Rohm and Haas Co., Philadelphia) was polymerized according to the method described in a technical report from the Rohm and Haas Co.[3] Low-molecular components were removed by dialysis following polymerization, and the polymer was isolated by alcohol precipitation. Alkaline hydrolysis of the primary amide groups of the polymethacrylamide (PMAA) was performed by the method of Arcus (3).

Results

Modification of Interaction by Physical Means. The hypothesis that gliadin molecules, possibly because of their high amide content, exhibit a marked molecular interaction through hydrogen bonding could be tested. Water is capable of bonding, through the hydrogen bridge, with other molecules containing groups having this property. Thus gliadin, in contact with water, imbibes water to form a semifluid hydrated phase. However, the competition of water with the interprotein hydrogen bonds is not strong enough to replace completely these intermolecular bonds, and the gliadin molecules remain *undis-*

[3] Technical Bulletin SP-32 (July 1953).

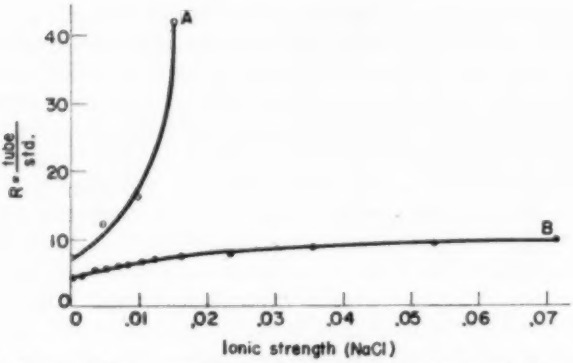

Fig. 1. Effect of ionic strength on turbidity of 1% gliadin solutions. Relative turbidity of solution to standard (benzene) versus ionic strength (of sodium chloride) in *A*, hydrochlo·ic acid-water solution, pH 3.6; *B*, hydrochloric acid-water-2*M* urea solution, pH 3.6.

solved in the water. Urea, formamide, and acetamide, among other possible hydrogen-bonding substances, can be more effective in competing with the gliadin intermolecular bonds than is water. Addition of urea, for example, to a gliadin-water suspension to the extent of only 1 to 2 molar concentration acts to dissolve the gliadin in the solvent. Urea, by bonding with the intermolecular hydrogen-bonding sites on the gliadin molecules, does so to such a degree that these sites are masked, statistically, to intermolecular cross-linking, and the gliadin molecules pass into solution in what approaches a molecular dispersion.

As noted earlier, the induction of a small electrical charge on the gliadin molecules, through an increase in intermolecular repulsion or an increase in osmotic attraction for the solvent, is also successful in sufficiently overcoming intermolecular cross-linking to allow the gliadin to dissolve in water. Addition of salts to such solutions serves to decrease the dispersing effect of the charge, and the intermolecular bonding again becomes predominant, with the gliadin precipitating as a hydrous mass from the solution.

a. Turbidity study: Such disaggregation-aggregation tendencies may be followed in their incipient stages by observation of the relative light-scattering properties of the system. Figure 1 illustrates the changes in turbidity, relative to a benzene standard (4), in the absence and in the presence of 2-molar urea; the changes are brought about by increasing the ionic strength, through sodium chloride addition, of a 1% solution of gliadin. The solution is prepared by titrating a salt-free gliadin-water suspension to pH 3.6 with hydrochloric acid.

The following inferences can be drawn from this experiment: 1) The dispersing effect of the charge induced through titration of the salt-free protein with hydrochloric acid to pH 3.6 is readily dissipated by the addition of salt at low ionic strength. 2) The presence of urea (2*M*) obviates the necessity of the charge in the dispersion process; the action of the urea is presumably that of masking the sites on the protein through which intermolecular cross-linking would occur. In fact, 2-molar urea will dissolve gliadin at any pH and ionic strength. 3) The low tendency of gliadin to disperse molecularly in water is due to hydrogen bonding between gliadin molecules. Water, as a hydrogen bonder, is weaker than the intermolecular hydrogen bonds of gliadin. Urea, in contrast to water, is a stronger hydrogen bonder and will effectively compete with and mask the intermolecular gliadin hydrogen bonds.

b. Electrophoresis study: On the basis of these inferences, it would be expected that the state of dispersion of gliadin at pH 3.8, 0.01 ionic strength, which are the experimental conditions of Schwert *et al.* (26), would approach molecular. If no residual protein-to-protein interaction exists under these conditions, electrophoresis patterns should reflect the existence or lack of existence of electrophoretic homogeneity, and the ascending and descending boundaries should be enantiographic in either case. Since the patterns obtained under these conditions are markedly nonenantiographic, it appears that the protein is indeed electrophoretically nonhomogeneous and that some residual interaction exists, as was postulated by Schwert *et al.* (26). Addition of urea to a 2-to-3-molar concentration should act to reduce the postulated interaction, and, in urea solution, the electrophoretic patterns should become, at least to a recognizable degree, more enantiographic. Figure 2 compares patterns obtained on a sample of crude gliadin (acetic acid preparation) at pH 3.8, ionic strength 0.01 in the absence of urea and in the presence of 2-molar urea, 6-molar urea, and 4-molar acetamide. Changes in the over-all appearance of the patterns are observed, but there is no real indication that they have become more enantiographic as a result of the action of urea.

At this point it was suspected that these anomalous electrophoresis patterns might be due to causes other than protein-protein interaction. It is known that electrophoresis patterns obtained on a wide variety of proteins, which appear to be electrophoretically homogeneous under other conditions, show anomalies under just the conditions at which it is possible to obtain gliadin in water solution, i.e. at very low ionic strengths and pH values close to the isoelectric point, where the protein bears only a small charge and where a high proportion

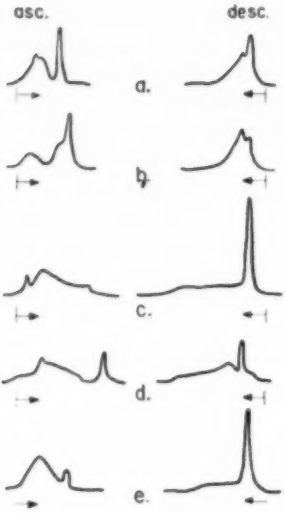

Fig. 2. Electrophoresis patterns of 1% gliadin in solution in various solvents at pH 3.8, ionic strength 0.01, 4°C. *a*, acetate buffer; field strength approximately 2.9 volts/cm, after 10,000 seconds; *b*, glycine-hydrochloric acid buffer; 2.7 volts/cm, 16,000 seconds; *c*, acetate buffer plus 6M urea; 6.5 volts/cm, 7,800 seconds; *d*, acetate buffer, 2*M* urea; 7.7 volts/cm, 6,400 seconds; *e*, acetate buffer, 4*M* acetamide; 2.9 volts/cm, 15,300 seconds. Protein positively charged.

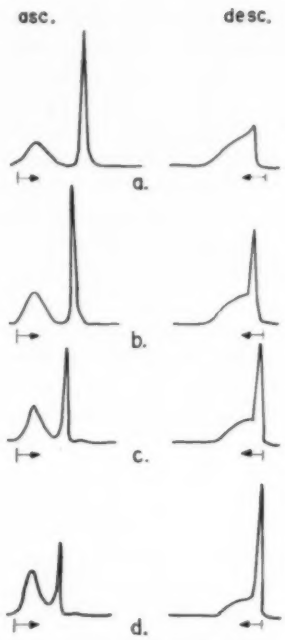

Fig. 3. Electrophoresis patterns of 1% bovine serum albumin and 1% ovalbumin in buffers of pH 4.4, ionic strength 0.01, 4°C. *a*, BSA in lithium-acetate buffer; field strength, 4.3 volts/cm, after 10,000 seconds; *b*, BSA in sodium-acetate buffer; 3.7 volts/cm, 10,000 seconds; *c*, OA in lithium-acetate buffer; 4.2 volts/cm, 10,000 seconds; *d*, OA in sodium-acetate buffer; 3.8 volts/cm, 10,000 seconds. Proteins positively charged.

of the conductivity of the solution is due to hydrogen ions present (Cann *et al.*, 7,8). Figure 3 illustrates patterns obtained with crystallized bovine serum albumin and 4x crystallized ovalbumin under these conditions of electrophoresis. The similarities to the patterns for gliadin at pH 3.8, ionic strength 0.01, are apparent. Figure 3 also illustrates variations in patterns related only to a change in the buffer cation with these proteins at 0.01 ionic strength. An increase in ionic strength of the solutions of these proteins (to ionic strength equal to 0.1 or greater), even at the same pH values, virtually eliminates the anomalies of the patterns, yielding instead, patterns of enantio-

graphic form and boundaries of degrees of heterogeneity or homogeneity similar to those obtained at neutral pH ranges for these proteins.

With gliadin, it is not possible to increase the ionic strength sufficiently to eliminate the anomalous patterns without precipitating the protein from solution. Figure 1, however, points to the possibility that, in urea solution, the ionic strength can be increased without a concomitant increase in the protein-protein interaction tendency. Electrophoretic patterns of 1% crude gliadin in 3-molar urea solutions of 0.1 ionic strength at pH values of 3.8 and 7.0 are illustrated in Fig. 4.

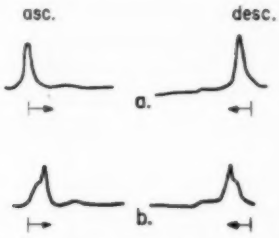

Fig. 4. Electrophoresis patterns of 1% gliadin in 0.10 ionic strength buffers plus 3-molar urea. *a*, phosphate buffer, pH 7.0; field strength, 4.6 volts/cm, after 14,400 seconds; migration toward (+) electrode. *b*, acetate-chloride buffer pH 3.8; 3.7 volts/cm, 12,600 seconds; migration toward (−) electrode.

The patterns are closely enantiographic for the ascending and descending boundaries. This verified the supposition that the anomalous patterns obtained with gliadin either in the absence or in the presence of urea at 0.01 ionic strength are not necessarily due to protein-protein interaction but are reflections of nonideal electrophoretic conditions which result in false boundaries and nonenantiography with many proteins. At pH 3.8, three peaks are evident on the patterns, indicating actual electrophoretic heterogeneity of the crude gliadin.

Conclusions to be drawn from these experiments are: 1) The interaction tendency of gliadin molecules with each other is probably of a hydrogen-bond nature. 2) At acid pH values ($<$ pH 4.0), the charge imposed on the gliadin molecules succeeds in counteracting their inherent hydrogen-bonding tendencies to such a degree as to allow molecular dispersion of the gliadin in aqueous solvents as long as the ionic strength is maintained very low ($<$ 0.02–0.03). 3) Urea in concentrations of 1- to 2-molar and above, by competing more strongly than does water for the hydrogen-bonding sites on the protein, allows the protein to pass into that which approaches molecular solution in the urea-water solvent. 4) Crude gliadin, at pH 3.8, is electrophoreti-

cally heterogeneous. 5) The nonenantiographic electrophoresis patterns obtained with gliadin at pH 3.8, ionic strength 0.01 (of Schwert *et al.*, 26) are not a result of residual interaction of electrophoretically nonhomogeneous components of the protein but, rather, a result of conditions of the experiment under which even homogeneous proteins show similar anomalies, the exact explanation of which is not yet available (32).

Modification of Interaction by Chemical Means. Since urea, formamide, acetamide, etc., seem to compete more successfully than water with the intermolecular hydrogen bonds of gliadin, it might logically be suspected that the high amide content of the gliadin molecule in itself is a source of the protein-protein interaction (through hydrogen bonds) that is characteristic of this protein. The absence of any appreciable charge on the gliadin molecules throughout the range of 2 to 3 pH units on either side of pH 7.0 would also be a contributing factor toward the effective existence of such intermolecular hydrogen bonding.

The amide groups on proteins are more readily susceptible to hydrolysis than are the peptide linkages. Mild hydrolyzing conditions can break off ammonia from the amide groups to a considerable degree without appreciable hydrolysis of the protein chain. Such mild and limited hydrolysis of the amide groups would result in two conditions that could modify the properties of the gliadin. First, a decrease in the number of amide groups could lower the intermolecular hydrogen-bonding capacity of the protein molecules. Second, the carboxyl groups simultaneously freed on the protein during amide hydrolysis, would, in pH regions where the COOH groups are ionized, increase the charge carried by the protein molecule. Both of these factors should act to increase the solubility and decrease relative interacting tendencies of the protein. A series of experiments was conducted to investigate these possibilities.

The course of hydrolysis of the amide groups of gliadin at three hydrochloric acid concentrations at 100°C. is illustrated in Fig. 5. The degree of hydrolysis is expressed in terms of the moles of ammonia released per liter of hydrolysis mixture containing 1 g. of gliadin per 100 ml. of solution. The hydrolysis appears to follow second-order kinetics.

No demonstrable hydrolysis of the peptide bonds of the protein accompanied the amide hydrolysis, even when the latter exceeded 50% of the total amide groups present. On a sample of gliadin from which 53.6% of amide had been removed, for example, no dialyzable or nonprotein nitrogen (unprecipitable with 8% trichloroacetic acid) other

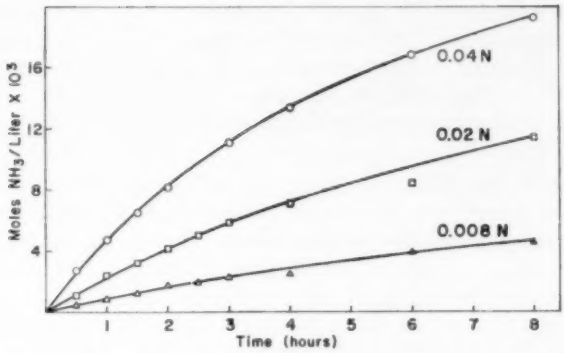

Fig. 5. Course of deamidization of gliadin (1% concentration) at 100°C. in hydrochloric acid solutions of concentrations indicated.

than ammonia could be found, and an ultracentrifuge pattern on the protein (pH 7.0 and 0.2 ionic strength phosphate buffer) showed a single boundary with an $s_{20,w} = 1.5 \times 10^{-13}$ sec. (Fig. 6, d).

Marked changes in solubility characteristics accompanied the hydrolyses, however. When less than about 5% of the amide nitrogen was removed, the protein retained its sticky character in contact with water at pH 7 and showed the solubility characteristics of gliadin at pH 3.8, 0.01 ionic strength. It was noted that, at pH 3.8, 0.1 ionic strength where gliadin is insoluble, smaller and smaller amounts of urea were required to induce solution of the hydrolysis product as the percent of amide removed was increased in this range. When the degree of amide hydrolyzed was greater than 10% of the total, the protein became insoluble in water at pH 3.8, 0.01 ionic strength, the precipitated protein was no longer sticky and hydrated, and the protein became readily soluble in water or buffer solutions at pH 7 and above. The electrophoretic properties of gliadin from which less than 5% of the amide groups had been removed and which was still soluble in the pH 3.8, 0.01 ionic strength, acetate buffer were similar to those of normal gliadin preparations.

Electrophoresis patterns run at pH 7.0, ionic strength 0.1 on the partially deamidized gliadin preparations soluble under these conditions showed only a single peak, indicating a fairly narrow distribution of electrophoretically different entities on either side of a mean, i.e. all molecules of the original gliadin had lost amide nitrogen to nearly the same extent (Fig. 6, a, b, and c).

From this group of experiments it is deduced 1) that the imposition of a charge on gliadin molecules is very effective in counteracting

interactions between the protein molecules and 2) that removal of amide groups, to the extent of 10% or more of the total, lowers the hydration tendency of the protein when in an insoluble state (e.g., the precipitate obtained with the partially deamidized protein at acid pH values) to such an extent that the doughlike property of the gliadin is lost.

Molecular Interaction Involving Amide Groups. In an attempt to identify the source of the distinctive properties of gliadin, a model substance of simpler constitution was sought. If these properties of glia-

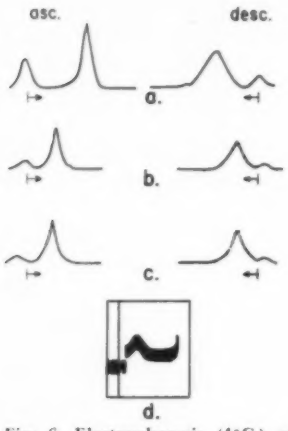

Fig. 6. Electrophoresis (4°C.) and ultracentrifuge (25°C.) patterns of partially deamidized gliadin. *a*, electrophoresis pattern for 53.6% amide groups removed; field strength 4.7 volts/cm, after 5,500 seconds. *b*, electrophoresis pattern for 15.6% amide groups removed; 4.1 volts/cm, 7,200 seconds. *c*, electrophoresis pattern for 10.9% amide groups removed; 4.1 volts/cm, 10,000 seconds. *d*, sedimentation pattern for preparation *a* after 154 minutes at 56,160 r.p.m. $S = 1.5 \times 10^{-13}$. *a*, *b*, and *c* in phosphate buffer pH 7.0 ionic strength = 0.10. *d*, same buffer, 0.20 ionic strength. Protein negatively charged.

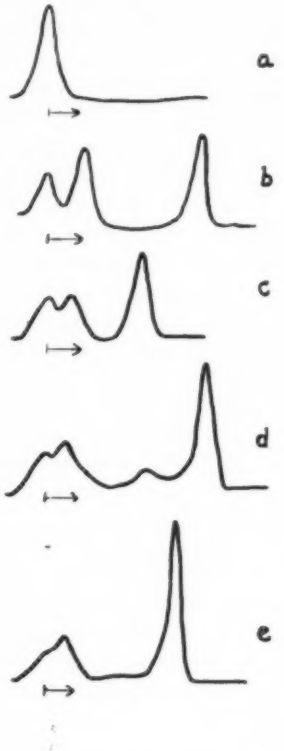

Fig. 7. Ascending boundary electrophoresis patterns of mixtures of uncharged polymethylacrylamide and charged molecules possessing amide groups in varying degrees. All were run in phosphate buffer of pH 7.0, 0.10 ionic strength at 4°C. and 4.1 volts/cm, for 10,000 seconds. *a*, 0.5% PMAA; *b*, 0.5% PMAA plus 0.5% partially deamidized PMAA (2.5% of amide groups removed); *c*, 0.5% PMAA plus 0.5% partially deamidized gliadin (15% amides removed); *d*, 0.5% PMAA plus 0.5% casein; *e*, 0.5% PMAA plus 0.5% ovalbumin.

din are related to the high amide content and to the low net charge of the protein in the pH regions where these distinctive properties are most in evidence, it could be expected that a high-molecular polyamide might show properties similar to those of gliadin. Such a polyamide was prepared from methacrylamide. Polymethacrylamide (PMAA) is a nonelectrolyte, and remains uncharged in contact with water; it contains some 1160 amide groups for 10^5 g. of polymer (compared to about 320 amide residues for 10^5 g. of gliadin). In contrast to gliadin, PMAA is readily water-soluble; i.e., water is able to compete successfully with any intermolecular hydrogen bonds in this substance, and no charge on the molecules is needed to accomplish this effect. To this extent PMAA is not a good model with which to study the properties characteristic of gliadin. However, if the amide groups do possess a tendency to be involved in intermolecular cross-links, that tendency would not be lessened by the presence of any charged groups in PMAA; a residual interaction tendency might be detectable in the solution of this polyamide.

On the assumption that the uncharged polyamide molecule will not be impeded in its tendency to approach in solution and to bond in some degree through hydrogen, with other amide-containing molecules which are themselves charged, it is possible to detect such bonding tendencies through electrophoresis studies on such mixed solutions.

The electrophoretic patterns shown in Fig. 7 give evidence of such interaction between components in solutions containing mixtures of PMAA with partially deamidated PMAA and in mixtures of PMAA with proteins which contain varying relative amounts of amide groups per unit weight.

This evidence arises from the presence of the asymmetry near the starting boundary in the ascending pattern. The uncharged PMAA component, which exhibits zero mobility when alone, is dragged along from the starting position through association with the mobile components. The extent of displacement of the nonmobile PMAA peak varies more or less directly with the amide content of the mobile component contained in these mixtures (partially deamidated PMAA — 1170 amide groups per 10^5 g., partially deamidated gliadin - 270, casein - 114, and ovalbumin - 72).

Although it is not possible to say unequivocally which functional groups of the proteins are involved in this interaction, the high amide group content of PMAA suggests that amide groups may play a significant part.

Particle Size Heterogeneity of Gliadin. The molecular weight and particle size distribution of a crude gliadin sample, prepared by the

acetic acid method (5), and of some of the thermal fractions prepared by the Haugaard and Johnson method (17) from this gliadin were determined by light-scattering and sedimentation-velocity experiments for comparison with the results of Krejci and Svedberg (18).

Preliminary experiments indicated that acetate buffer pH 3.5, ionic strength 0.10 (0.05*N* sodium acetate, 0.05*N* sodium chloride), in 3*M* urea, was a suitable solvent for these experiments. A 1% solution of the crude gliadin preparation in this buffer possessed noticeable turbidity which was not removed by pressure filtration through an ultrafine sintered glass filter. Light-scattering measurements on this filtered solution of crude gliadin (an acetic acid preparation) showed a linear Hc/τ vs. c plot and indicated a weight average molecular weight of about 250,000. The ultracentrifugal pattern of this crude gliadin in 65% alcohol showed a main peak, $s_{20,w} = 1.8 \times 10^{-13}$ sec, which constituted about 90% of the total protein. The remainder of the pattern indicated the presence of a spectrum of heavier materials (Fig. 8, a). A similar distribution of material was shown in the ultracentrifuge pattern of the crude gliadin in the 3*M* urea-acetate buffer solution.

Ultracentrifugation for 2 hours at 42,000 r.p.m., in preparatory rotor A of the Model E Spinco analytical-type apparatus, clarified the original 1% solution of gliadin in the acetate-3*M* urea buffer and yielded three fractions: a residue of gummy material, a clear solution, and a very turbid layer (probably lipid material) of lower density than that of the solution. This lipid fraction is believed to be the main contributor to the turbidity of crude gliadin solutions. Careful removal of the clear solution with a syringe allowed a molecular weight determination on this dissolved gliadin fraction by light scattering. Results yielded a value of 110,000 (Fig. 9, D). The sedimentation pattern obtained on this preparation contained a homogeneous main peak representing over 95% of the area with a sedimentation constant, $s_{20,w}$ of 1.82×10^{-13} sec (Fig. 8, b).

Fractions F_2 and F_3 from the thermal fractionation were dissolved in the acetate-urea buffer and examined in the ultracentrifuge and by light scattering. Fraction F_4 was suspended in water and brought into solution with hydrochloric acid alone at pH 3.5; ultracentrifugation and light-scattering measurements were made on this salt-free solution. Two observations with regard to the light-scattering data are worthy of comment and deserving of further study. First, specific refractive index increments ($\Delta n/c$, see Table I) of the gliadin samples increase significantly as impurities are removed. This could reflect the progressive removal of some poorly refracting substance such as a lipid or lipoprotein contaminant. Second, in the Hc/τ versus c curves (Fig. 9), it

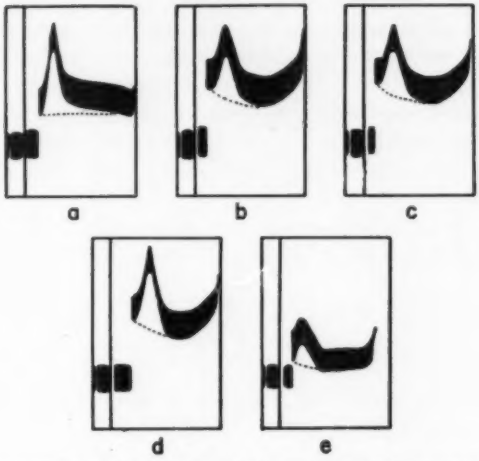

Fig. 8. Ultracentrifuge patterns of gliadin and of gliadin fractions. *a*, 1% crude gliadin in 65% alcohol-phosphate buffer pH 6.6, 0.01 ionic strength after 157 minutes at 56,160 r.p.m., temp. 24.3°C.; *b*, 1% ultracentrifugally purified gliadin in acetate buffer pH 3.5, 0.10 ionic strength, 3*M* urea, after 144 minutes at 59,780 r.p.m., temp. 26.8°C.; *c*, 1% fraction F_2, conditions as for *b*, except temp. 27.6°C.; *d*, 1% fraction F_3, conditions same as for *b*; *e*, 1% fraction F_4 in hydrochloric acid-water solution pH 3.6 after 92 minutes at 56,160 r.p.m., temp. 23.7°C.

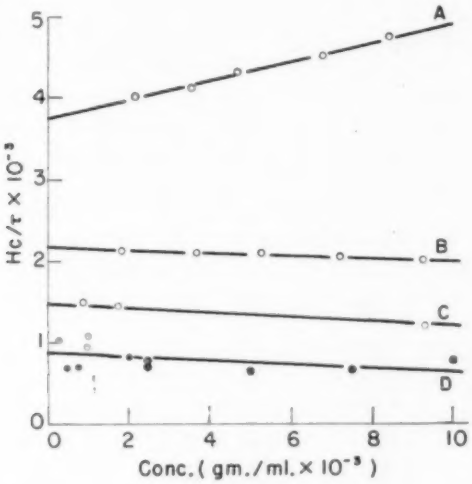

Fig. 9. Light-scattering data on gliadin fractions. *A*, fraction F_4 in solvent C (Table I); *B*, fraction F_3 in solvent B (Table I); *C*, fraction F_2 in solvent B; *D*, ultracentrifugally purified gliadin in solvent B.

TABLE I

ULTRACENTRIFUGE AND LIGHT-SCATTERING DATA ON GLIADIN AND GLIADIN FRACTIONS

GLIADIN SAMPLE	ULTRACENTRIFUGE				LIGHT SCATTERING		
	Solvent[a]	$s_{20,w} \times 10^{13}$	Approximate Percent of Total Area Under Main Peak	M_s[b]	Solvent	$\frac{\Delta n}{c} \times 10^5$	M_{LS}
Crude	A	1.8	90	24,000	B		250,000
Ultracentrifuge-purified	B	1.82	95	24,000	B	0.153	110,000
F_2	B	1.82	98	24,000	B	0.173	67,000
F_3	B	1.77	100	23,500	B	0.178	45,000
F_4	C	1.35[c]	100	....	C	0.184	26,500

[a] Solvent A = 65% aqueous ethyl alcohol, 0.01 ionic strength with phosphate buffer pH 6.6. Solvent B = acetate buffer pH 3.5, ionic strength 0.1, 3*M* urea. Solvent C = water with just enough HCl to bring pH of solution to 3.5.

[b] Molecular weight calculated from observed value of $s_{20,w}$, and literature values for diffusion constant ($D_{20,w} = 6.72 \times 10^{-7}$) (see ref. 18) and partial specific volume of the protein ($V_{20,w} = 0.722$) (see ref. 18).

[c] The low value of $s_{20,w}$ in this solvent probably is due to a Donnan effect, which would become important with the charged state of the protein and at the very low ionic strength of this solution. No value of M_s is calculated because literature value for diffusion constant cannot be assumed to hold under these conditions.

is apparent from the slopes that the water-HCl solvent (positive slope) is a "better" solvent (wherein less attractive forces act between the protein molecules) than the 3*M* urea, pH 3.5, 0.1 ionic strength acetate buffer solvent (slightly negative slopes). It seems probable, from this evidence, that complete molecular dispersion of the protein (i.e., complete elimination of protein-to-protein interaction) may be more closely approached in the former than in the latter of these solvents (15). Light-scattering measurements on the same samples of the protein in each of these solvents will need to be made to determine the influence that this circumstance may have upon the apparent molecular weights obtained by this method. It should be emphasized, too, that the molecular values reported from the ultracentrifuge data have been calculated on the assumptions that the diffusion constants and partial specific volumes of the protein are constant as between samples and are equal to the values used by Krejci and Svedberg (18). The molecular values reported for both the ultracentrifuge and light-scattering methods are to be regarded, therefore, as of an exploratory rather than an exact nature.

The sedimentation patterns and Hc/τ vs. c plots for these fractions are given in Figs. 8 and 9 respectively. The results of these experiments, summarized in Table I, support the following conclusions: 1) Crude gliadin consists of a component, homogeneous with respect to sedimentation properties and having an $s_{20,w}$ value of about 1.8×10^{-13} sec, which makes up approximately 90% of the weight of the preparation. The remaining 10% consists of a heterogeneous mixture of materials (protein), which sediment at higher rates than the 90%

component, plus a fraction having a density less than that of proteins generally, which probably is of a lipid or lipoprotein nature. These impurities contribute in large degree to the light-scattering properties of the crude gliadin preparation. 2) Fractionation processes which involve successive partial precipitations of the true or 90% gliadin component succeed in the eventual removal of the impurities and yield a final small percentage of the true gliadin in such a purified state that agreement is reached between molecular weights determined by ultracentrifugal and light-scattering procedures. The molecular weight of the true gliadin component obtained by both methods is approximately 25,000.

Discussion

Gliadin, in contact with water, imbibes or combines physically with a considerable amount of water and yields the doughlike, hydrated mass characteristic of the protein at and near neutral pH values. This hydration tendency of the gliadin molecules is, however, not sufficient to overcome an inherent intermolecular attraction, probably through hydrogen bonds involving the amide groups on the protein, and thus not sufficient to allow the gliadin to disperse molecularly in the aqueous solvent. Imposition of an electrical charge on the gliadin molecules, as by titration to pH values below 4, shifts the balance of hydration versus intermolecular binding tendencies in favor of the former, and the protein passes into what approaches molecular dispersion in water. A small increase in ionic strength of such a solution neutralizes the charge effect, and the gliadin precipitates as a hydrated mass again. Independent of this peptizing action of a charge on the protein molecule, addition of substances such as urea, formamide, acetamide, which are stronger hydrogen bonders than water, serve, through competition with the intermolecular hydrogen bonds in the protein mass, to lower this intermolecular cross-linking tendency and to promote the passage of the protein into dispersion or solution. In a 2- to 3-molar solution of urea, for example, the intermolecular attraction of gliadin is so completely nullified that molecular or nearly molecular dispersions are attained in the urea-aqueous solvent independent of the pH and ionic strength of the solution. Molecular kinetic properties of gliadin can be studied under such conditions with minimal interference from intermolecular aggregation tendencies in the solution.

Other conditions under which gliadin can be obtained in a state of dispersion approaching molecular are: 1) at acid pH below about 4.0 and in total absence of salts or electrolytes other than the acid

required to titrate the protein solution to that pH; as the ionic strength is increased from this state, aggregation increases rapidly (through stages of polymolecular aggregates) until above about 0.03 ionic strength the gliadin is totally reprecipitated; 2) in 65–70% alcohol, molecular dispersion of the gliadin is approached; the effect in this case is possibly through competition of the alcohol with the intermolecular hydrogen bonds of the protein, similar to that postulated for the action of urea.

In any case, the approach to molecular dispersion is a statistical effect, and some residual interaction may always exist. The impurities present in the acetic acid and in the alcohol preparations of gliadin probably result from this residual interaction of gliadin with molecules of the gluten mixture other than gliadin; the percentage of such impurities present in a given preparation should vary with the degree to which gliadin intermolecular attractions are suppressed under the conditions of preparation.

The anomalous electrophoretic patterns for crude gliadin at pH 3 to 4, ionic strength 0.01, which have been interpreted (26) as reflecting a residual interaction of the protein molecules, including the corollary requirement that the protein must be electrophoretically heterogeneous, are undependable in this regard because of the nonideal conditions of the electrophoresis. It is observed that other proteins under similar solvent conditions show similar anomalies. It is, then, the conditions of the solvent system, not heterogeneity of the protein or any interaction that may exist between the protein components, that are primarily responsible for the observed anomalous patterns.

When crude gliadin is examined electrophoretically under conditions which minimize the nonideal solvent composition, as for example, at 0.1 ionic strength in 3-molar urea, crude gliadin appears to be heterogeneous (three peaks) at pH 3.8, where an appreciable charge exists on the component molecules; however, the patterns are enantiographic, which indicates that under these conditions no appreciable interaction of the components is occurring. Whether purified or true gliadin is electrophoretically heterogeneous unfortunately was not determined.

Partially deamidized crude gliadin, after 10–50% of the amide nitrogen has been removed under conditions accompanied by no detectable peptide bond hydrolysis, shows, electrophoretically, only a statistical heterogeneity indicated by a spreading of a single peak greater than that due to diffusion. A marked change in solubility characteristics accompanies the deamidization, however; the deamidized

gliadin becomes soluble at neutral pH range with little or no effect of changes in ionic strength. Deamidized gliadin shows minimum solubility in the acid region of pH, where native gliadin becomes soluble in the absence of salts. Precipitated deamidized gliadin has lost the hydration capacity which is characteristic of native gliadin under conditions where it is insoluble and which is essential to the dough-forming properties of the native gliadin. In ultracentrifugal analysis, a 50% deamidized gliadin shows little change in the sedimentation constant from that of the major fraction of the native protein.

Fractional precipitation of crude gliadin from 60% alcohol solutions results in a gradual elimination of the impurities and leaves a purified fraction (in very low yield) of the true gliadin in the supernatant, which yields comparable molecular values of about 25,000 by the ultracentrifuge and by light-scattering methods. The impurities, which are responsible for the abnormal turbidities of crude gliadin and which are eventually eliminated by successive precipitational fractionation, are partially of a protein nature and partially of a lipid or lipoprotein nature (24).

That the intermolecular attractions in gliadin are of a hydrogen-bond nature is indicated by their susceptibility to fission by urea. That the amide groups of gliadin may constitute important sites for the intermolecular hydrogen bonds is given support by the finding that an electrically neutral polyamide, polymethacrylamide, in a system containing charged proteins, is carried along by the electrophoretically mobile protein components to an extent roughly proportional to the amide content of the mobile component.

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GRAIN STORAGE STUDIES

XXVIII. The Influence of Temperature and Moisture Level on the Behavior of Wheat Stored in Air or Nitrogen[1]

R. L. Glass, J. G. Ponte, Jr., C. M. Christensen, and W. F. Geddes

ABSTRACT

Sound wheat (97% germination) was stored in atmospheres of air or nitrogen (containing 0.04% oxygen), at temperatures of 20° and 30°C., and at moisture contents ranging from 13 to 18%. Samples were removed at intervals of 2 to 4 weeks and tested for mold counts, viability, germ damage, fat acidity, and reducing and nonreducing sugars.

In grain stored at 30°C. the onset of deterioration as measured by the above criteria was delayed somewhat by storage in nitrogen as compared with storage in air. Once deterioration had begun in the grain stored in nitrogen, it progressed about as rapidly as in the grain stored in air.

At 20°C., deterioration was much slower in the samples stored in nitrogen than in those stored in air. Viability of the grain stored in nitrogen remained fairly high for 16 to 24 weeks, even at 18% moisture content, and germ damage developed much more slowly than in the samples stored in air. At 16% moisture, a No. 2 composite dark northern spring wheat stored in nitrogen was free of germ damage after 40 weeks, and a high-grade Marquis wheat after 48 weeks, whereas the same wheats stored in air had respectively 100 and 95% germ damage.

Storage in nitrogen prevented mold growth at all moisture levels, but this did not prevent decreases in nonreducing sugars, increases in fat acidity, or death and discoloration of the seed embryos, particularly at 30°C.

It is concluded that at 30°C. inert storage is not feasible for damp wheat, owing to a very active seed metabolism. At 20°C., the wheat enzymes are sufficiently inactive so that, together with the exclusion of mold growth by a nitrogen atmosphere, some benefit might be derived from this type of storage.

Fungi are largely responsible for deterioration of grain stored at moisture contents of 14 to 18%, and a number of workers have thought that spoilage of moist grain might be prevented if the grain were stored in carbon dioxide or nitrogen to prevent the growth of fungi.

Swanson (13) stored wheat in sealed containers at room temperature for prolonged periods of time. He observed that mold growth was essentially prevented after early mold activity had depleted the oxygen supply, with a resulting accumulation of carbon dioxide. However, damage to the wheat, as shown by all criteria, progressed steadily although somewhat more slowly than in control samples stored in an abundance of air.

Milner *et al.* (9) stored wheat at 18% moisture in atmospheres of carbon dioxide, nitrogen, and oxygen respectively at room tempera-

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ture for a period of 6 months. "Sick" wheat developed in all three samples at about the same rate. At the end of 5 months all samples were found to contain internal molds and bacteria. This last observation was surprising in view of the reports that bacteria are not expected to grow at relative humidities below 95% (10) and the equilibrium relative humidity for wheat at 18% moisture is approximately 86%. Matz and Milner (8) held dampened wheat in air and nitrogen for 16 weeks and concluded that anaerobic conditions were more damaging to baking quality than storage in an oxygen atmosphere.

Essentially the same results were obtained by Bottomley *et al.* (3), who stored corn at various temperatures, humidities, and oxygen concentrations. They reported that mold growth and various biochemical properties of the corn were affected more by increasing moisture contents than by reducing the oxygen concentration from 21 to 0.1%.

Oxley (11) stored wheat in bulk at moisture contents up to 24% in sealed silos. The storage life of the grain was lengthened because of accumulation of carbon dioxide, although the flour milled from the wheat had a distinct sour odor and rather marked changes occurred in some of the biochemical properties of the wheat.

This paper presents the results obtained when small samples of wheat were stored at varying moisture contents in air and nitrogen respectively, at 20° and 30°C., and examined periodically for degree of mold infestation, viability, and other indexes of deterioration.

Materials and Methods

Wheat. Two lots of wheat were used; one was very high quality certified seed of the variety Marquis, grown in Montana, the other a composite of a number of commercial samples of grade No. 2 dark northern spring. Both were bright, free of germ damage, and germinated 97%. The wheats were divided into sublots of 700 g. each which were then placed in 1-liter round-bottomed flasks and adjusted to various moisture contents ranging from 13 to 18% by addition of calculated amounts of distilled water. The flasks were held at room temperature for 24 hours and shaken frequently to allow for the equilibration of moisture.

All flasks were fitted with ground-glass connections, which permitted convenient aeration and withdrawal of samples of the interseed gas for analyses. Those samples to be stored under nitrogen were then flushed with tank nitrogen, which contained 0.4% oxygen and which was brought to the proper relative humidity for the particular wheat moisture level by passing it through aqueous sulfuric acid solutions of the required specific gravity (14).

The flasks were immersed in a water bath, held at 30°C. in one experiment and at 20°C. in the other. At 24-hour intervals a sample of the interseed air was removed from each flask for analyses. The flasks were then flushed with humidified air or nitrogen and returned to the water bath.

At intervals of 2 to 6 weeks, samples were removed for testing.

Analytical Methods. Interseed gases were analyzed for carbon dioxide and oxygen with a Haldane-Henderson apparatus according to the method of Peters and Van Slyke (12).

The volume of gas space present in each flask when filled with wheat was previously determined. This volume was corrected during the course of the experiment to allow for the changes which occurred as wheat was removed, so that the total daily production of carbon dioxide could be calculated. Results are expressed as mg. of carbon dioxide produced by 100 g. of wheat (dry-matter basis) in 24 hours.

Moisture contents were determined by the two-stage air oven method (1).

Seed viability was determined by the Minnesota State Seed Testing Laboratory. One hundred seeds were placed between damp blotting papers and held at 20°C. Those seeds which had normal shoots after 7 days were considered viable.

Fat acidity was measured on a diethyl ether extract (6 hours) of 3 g. of ground meal (Micro-Wiley mill, No. 40 screen) according to the method of Ames and Licata (2) as modified by Hunter *et al.* (7). The results are expressed as mg. potassium hydroxide required to titrate the fatty acids obtained from 10 g. dry wheat.

Reducing and nonreducing sugars were determined by the alkaline ferricyanide method (1) and are expressed as mg. of maltose and sucrose, respectively, per 10 g. of dry wheat.

Germ damage was determined by the Grain Inspection Division, U.S. Marketing Service, Minneapolis. This consisted of peeling back the pericarp covering the germ and examining for germ discoloration.

Mold counts were made by comminuting 5 g. of grain in 500 ml. of sterile 0.15% agar solution in a Waring Blendor; 5-ml. aliquots were further diluted in the same suspension medium and replicate 1-ml. portions cultured in malt-salt agar (4).

Experimental Milling and Baking Tests. At 12-week intervals a 150-g. sample of wheat was tempered to 15% moisture and milled on a two-stand micro mill similar to that described by Geddes and Frisell (5). Flour yield was expressed as the percentage of flour represented in the total mill products which were recovered.

Micro baking tests were carried out, using essentially the method

described by Geddes and Sibbitt (6). Flour (25 g.) was mixed with 0.75 g. yeast, 1.25 g. sucrose, 0.5 g. salt, 0.5 mg. potassium bromate, and the appropriate amount of water for the time necessary to obtain satisfactory dough development. The dough, scaled to 40 g., was fermented for 2 hours at 30°C., molded, proofed for 55 minutes, and baked at 232°C.

Results

Storage Trials at 30°C. Two separate experiments were made at 30°C. In the first trial the samples (Marquis) were stored at moisture levels which ranged from 13 to 18% in 1% increments and in air or nitrogen. The grain was sampled at 6-week intervals for a total of 42 weeks at moisture contents of 13 and 14%; 36 weeks at 15 and 16%; and 24 weeks at 17 and 18%. In the second trial at 30°C. the wheat (Marquis) was stored at moisture contents of 15, 16, 17, and 18%, in

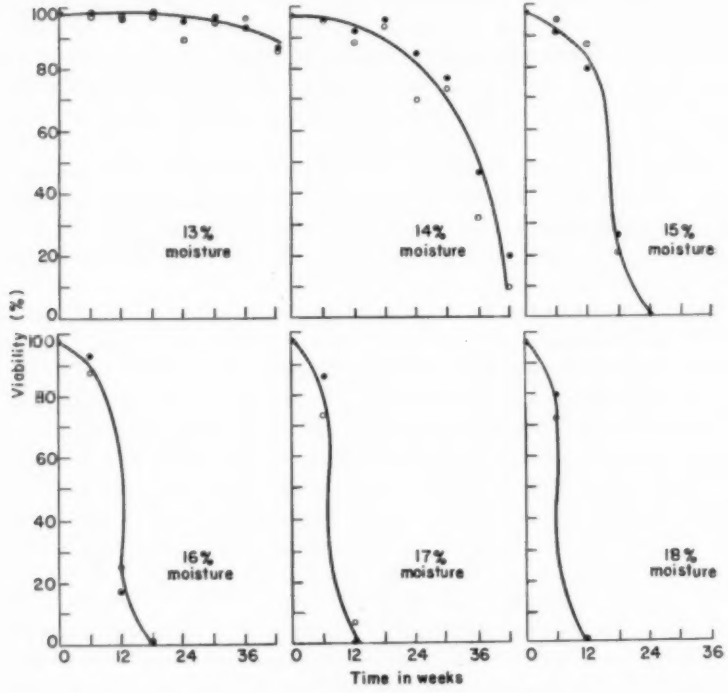

Fig. 1. Changes in viability of Marquis wheat stored at different moisture contents in an atmosphere of air (open circles) or nitrogen (solid circles) at 30°C.

air or nitrogen, for a total of 16 weeks with sampling intervals of 2 weeks.

First Trial at 30°C. Viability: Storage under an atmosphere of nitrogen was without effect in preventing or noticeably delaying a loss in viability, as is shown in Fig. 1. As the moisture content was increased from 13%, viability decreased rapidly and at 18% moisture no viable seeds remained in the wheat stored 12 weeks in either air or nitrogen.

Odor: Very pronounced odors developed in all samples stored at 17 and 18% moisture by the end of the eighteenth week. Those stored under nitrogen had particularly noticeable sour odors characteristic of fermentation.

Fat acidity: Fat acidity increased in all samples (Fig. 2). The rate of increase was more rapid at the higher moisture contents, and particularly so in the samples stored in air. At 16 to 18% moisture, there was approximately twice the amount of free fatty acids at the end of 24 weeks in the sample stored in air.

Reducing and nonreducing sugars: The changes which occurred in the sugars during the first 24 weeks of the experiment are summarized in Fig. 3. Initially, the wheat contained 37 mg. reducing sugars (expressed as maltose) and 232 mg. nonreducing sugars (expressed as su-

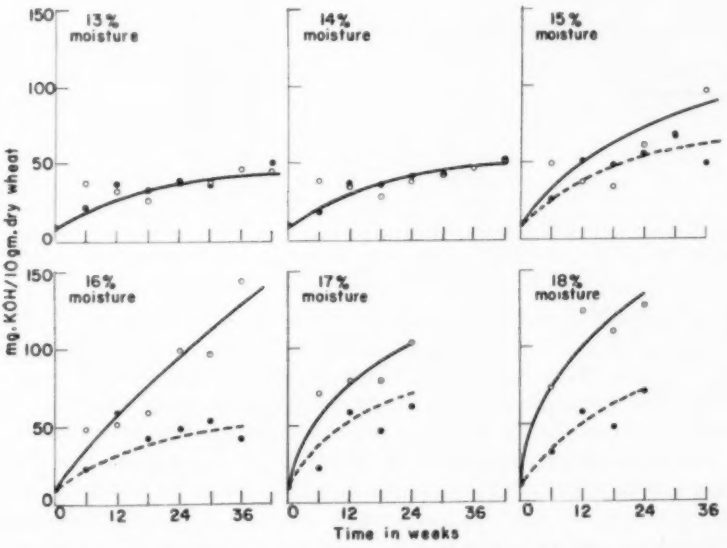

Fig. 2. Changes in fat acidity of Marquis wheat stored at different moisture contents in an atmosphere of air (open circles) or nitrogen (solid circles) at 30°C.

crose) per 10 g., dry-matter basis. At all moisture contents an increase in reducing sugars was observed. This change was more marked at the higher moisture contents, particularly so in the case of those samples stored in nitrogen. At 18% moisture there was a 285% increase in reducing sugars in the nitrogen-stored sample as compared with 120% in the air-stored sample. Nonreducing sugars decreased in all samples above 13% moisture and, again, this change was somewhat greater at the higher moisture contents, although in this experiment the air-stored samples showed a greater decrease than those stored in nitrogen.

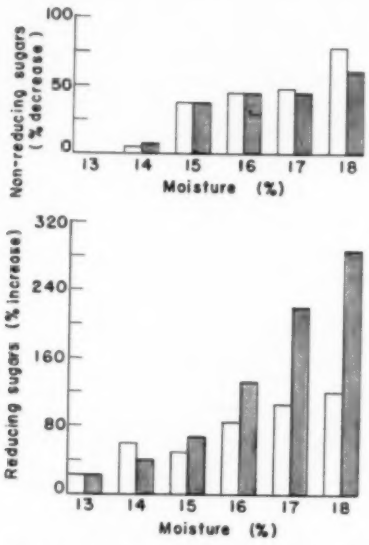

Fig. 3. Changes in reducing and nonreducing sugar contents of Marquis wheat stored at different moisture contents in an atmosphere of air (open bars) or nitrogen (cross-hatched bars) at 30°C. for 24 weeks.

Mold growth and carbon dioxide production: Mold count (Table I) increased rapidly at moisture contents of 15% and above in the grain stored in air, but the grain stored in nitrogen remained virtually mold-free. The fungi present were principally species of *Aspergillus* and *Penicillium* known to invade grain at these moisture contents.

Carbon dioxide production (Table II) was much greater in samples stored in air, at 15% moisture and above, than in samples stored in nitrogen. In air, the production of carbon dioxide increased throughout the storage period; in nitrogen more than half of the carbon dioxide was produced during the first 6 weeks of storage.

TABLE I

EFFECT OF ATMOSPHERE, MOISTURE CONTENT, and STORAGE TIME AT 30°C. ON MOLD COUNT OF MARQUIS WHEAT

Storage Time	Mold Count[a] per Gram (in Thousands)					
	Moisture Content, Per Cent					
	13	14	15[b]	16[b]	17[c]	18[c]
weeks						
	Stored in air					
6	0.5	6.0	4	180	360	630
12	0.1	2.6	360	3,120	2,940	24,200
18	0.4	0.3	2,180	4,700	3,200	42,200
24	0.0	3.8	2,400	3,800	6,800	38,000
30	0.3	0.3	2,850	4,100		
36	0.0	0.0	340	46		
42	0.3	3.5				
	Stored in nitrogen					
6	0.0	0.0	0.0	0.0	0.0	0.0
12	← less than 0.1 →					
18	← less than 0.1 →					
24	← less than 0.3 →					
30	← less than 0.3 →					
36	← less than 0.3 →					
42	0.3	2.5				

[a] Original seed gave a mold count of 500 per g.
[b] Discontinued at the end of 36 weeks.
[c] Discontinued at the end of 24 weeks.

TABLE II

EFFECT OF ATMOSPHERE AND MOISTURE CONTENT ON THE AVERAGE WEEKLY CARBON DIOXIDE PRODUCTION OF MARQUIS WHEAT[a] STORED AT 30°C. FOR 24 WEEKS

Storage Atmosphere	Carbon Dioxide Production at Moisture Content, Per Cent					
	13	14	15	16	17	18
Air	0.25	0.54	17.0	27.7	54.0	115.0
Nitrogen	0.21	0.25	0.54	0.75	1.8	3.9

[a] Expressed as mg. carbon dioxide produced per 100 g. dry wheat per week for 24 weeks.

Germ damage: Germ damage developed earlier in the samples stored in air at 16 to 18% moisture (Table III). After 12 weeks the germs of the air-stored samples were very extensively darkened, while the nitrogen-stored samples were virtually free of damaged germs. These differences had largely disappeared after 18 weeks of storage. The development of "sick" wheat was much slower at 15% moisture and was nonexistent at 13 and 14% moisture.

Milling and baking tests: Milling and baking tests were carried out on samples taken at 12, 24, and 36 weeks. No evidence of deterioration could be detected in the milling properties of the various samples. The flour yields remained the same for all samples throughout the storage experiment.

TABLE III

EFFECT OF ATMOSPHERE, MOISTURE CONTENT, AND STORAGE TIME OF WHEAT AT 30°C. ON DEVELOPMENT OF GERM DAMAGE

Storage Time	Germ Damage[a] at Moisture Content, Per Cent					
	13	14	15	16	17	18
weeks						
	Stored in air					
6	0	0	0	0	3	7
12	0	1	4	40	60	87
18	0	0	4	52	99	100
24	0	0	13	3	100	100
30	0	0	30	75		
36	0	T	75	85[b]		
42	0	0				
	Stored in nitrogen					
6	0	0	0	0	0	0
12	0	3	0	5	7	4
18	2	3	6	20	95	100
24	0	0	4	57	100	100
30	0	0	10[c]	98		
36	0	0	65	100		
42	0	0				

[a] Original sample had no germ damage. Determinations were made by the Federal Grain Supervision Office.
[b] Marginal damage 15% additional.
[c] Marginal damage.

TABLE IV

EFFECT OF ATMOSPHERE, MOISTURE CONTENT, AND STORAGE TIME ON THE BAKING QUALITY OF MARQUIS WHEAT (Wheat stored at 30°C.)

Moisture	Loaf Volume		
	Storage Time		
	12 Weeks	24 Weeks	36 Weeks
%	*cc*	*cc*	*cc*
	Stored in air		
13	134	139	152[a]
14	137	140	146[a]
15	120	116[a]	138[a]
16	120	112[a]	128[a]
17	90	...[b]	...[b]
18	...[b]	...[b]	...[b]
	Stored in nitrogen		
13	139	139	141
14	137	130	134
15	141	120	125
16	122	103	100
17	86	...[b]	...[b]
18	72	...[b]	...[b]
Original[c]	155	145	153

[a] The flour and bread crumb were dark gray.
[b] Not baked because of extremely poor dough-handling qualities.
[c] The original wheat was stored at −10°C.

Baking tests (Table IV) showed very marked deterioration in flour quality when the wheat was stored at the higher moisture contents in either air or nitrogen. At the end of 12 weeks the flour from wheats stored in air at 18% moisture gave a dough of such inferior handling properties that it could not be baked; milling and baking tests were therefore not made on these samples at the 24-week period. After 24 weeks the wheats stored at 15 and 16% moisture had deteriorated considerably and the flours from the air-stored wheats were quite gray in color, as were the crumbs of the resulting breads. All of them had marked odors which persisted in the bread. After 36 weeks the flours from the wheats stored at 13 and 14% moisture still gave loaves of satisfactory volumes, but those stored in air had a gray crumb color.

Second Trial at 30°C. In the tests which have been discussed, the

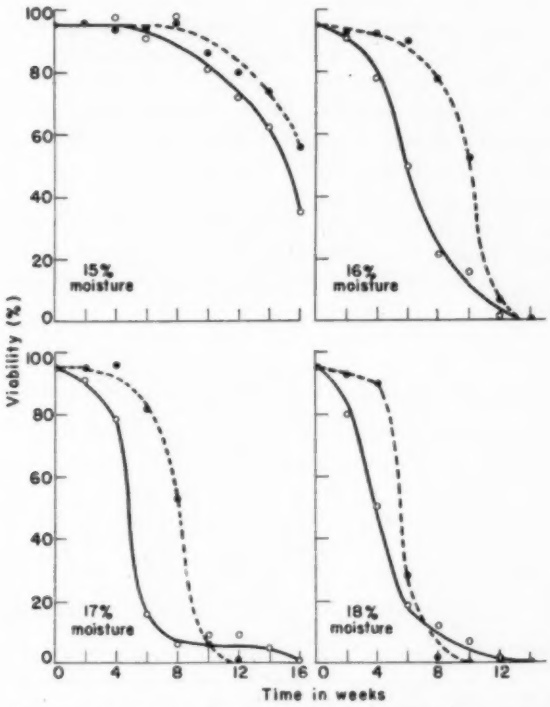

Fig. 4. Changes in viability of Marquis wheat stored at different moisture contents in an atmosphere of air (open circles) or nitrogen (solid circles) at 30°C.

wheat stored at moisture contents of 15% and above deteriorated too rapidly for the course of deterioration to be followed accurately with sampling only every 6 weeks. The test was repeated, therefore, with Marquis wheat at moisture contents of 15, 16, 17, and 18%, and samples were taken every 2 weeks for 16 weeks. The viability of all samples decreased markedly during storage (Fig. 4) for 16 weeks. At 16 to 18% moisture the samples in air began to deteriorate 2 to 4 weeks earlier than did the nitrogen-stored samples. The differences did not persist, however, and within a short time the viability of the nitrogen-stored as well as the air-stored wheat had decreased to zero.

A summary of the other changes which occurred in this wheat during the 16-week storage period is given in Table V. At all moisture contents fat acidity increased more slowly in the nitrogen-stored sample than in the corresponding air-stored sample. Reducing sugars, on the other hand, increased more rapidly in the wheat stored in nitrogen. The final value in the wheat at 18% moisture was 192% higher than the initial value. In contrast, reducing sugars increased only 30% in the wheat stored in air at this moisture content. Nonreducing sugars decreased faster in the wheat stored in air. At all moisture levels mold growth was effectively inhibited by the atmosphere of nitrogen but was very extensive in air. The differences in mold activity were again reflected in the large differences in carbon dioxide production in the two atmospheres at the various moisture levels.

Storage Trials at 20°C. The storage trials at 20°C. were made at 14, 16, and 18% moisture using both the Marquis and dark northern spring composite No. 2. The samples were removed for analyses at 4-week intervals.

TABLE V

CHANGES OCCURRING IN MARQUIS WHEAT STORED UNDER AIR AND NITROGEN AT 30°C. FOR 16 WEEKS

	Moisture Content							
	Air				Nitrogen			
	15%	16%	17%	18%	15%	16%	17%	18%
Free fat acidity[a]	36.0	77.4	93.2	103.0	35.0	35.9	41.1	42.1
Reducing sugars[b]	32	44	44	48	38	52	83	108
Nonreducing sugars[c]	186	133	127	98	188	176	144	129
Mold count[d]	0.7	1.4	10.6	40	0	0	0	0
Carbon dioxide production[e]	9.4	51.1	109.2	204.0	1.0	2.6	6.6	13.9

[a] Initial free fat acidity 27 mg. KOH per 100 g. dry wheat.
[b] Initial value 37 mg. maltose per 10 g. dry wheat.
[c] Initial value 232 mg. sucrose per 10 g. dry wheat.
[d] Mold count times 10^{-6}.
[e] Milligrams of CO_2 produced per 100 g. wheat (dry basis) per week.

Viability: Germination tests, summarized in Fig. 5, showed that at 16 and 18% moisture a delay in loss of viability occurred in the samples stored in nitrogen. The difference in the rate of loss in viability became more pronounced as the moisture content was increased and was also more apparent in the higher-quality wheat (Marquis). This wheat, when stored at 18% moisture, remained sound for 16 weeks in nitrogen but began to lose viability rapidly in an atmosphere of air. After 8 weeks the latter contained only 18% viable seeds, while the nitrogen-stored sample remained at about the original value of 97% viable seeds until the end of 20 weeks, at which time viability was 80%. The storage of wheat in air at 18% moisture was discontinued at 24 weeks because of extensive deterioration which had taken place.

Fat acidity: Storage in nitrogen greatly retarded an increase in fat acidity (Fig. 6), especially in the Marquis wheat at 18% moisture, and in the No. 2 dark northern spring at 16 and 18% moisture. Fat acidity

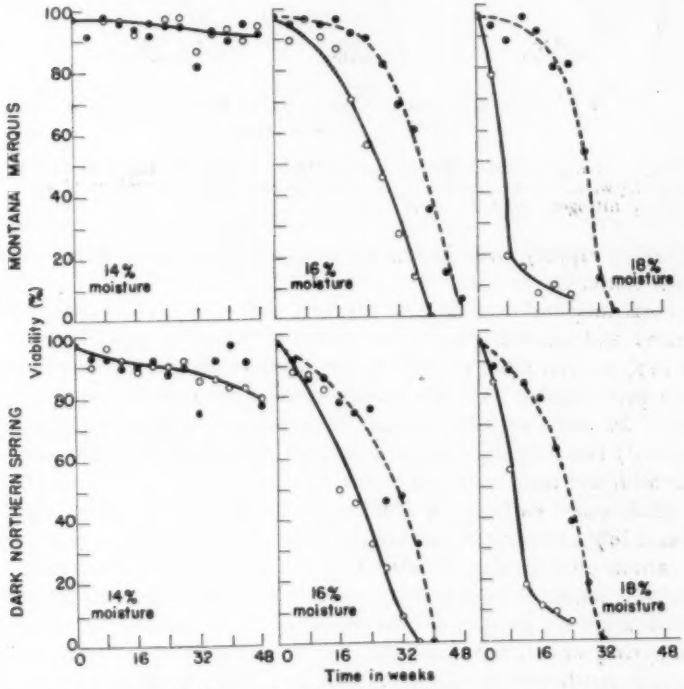

Fig. 5. Changes in viability of Marquis and a composite sample of dark northern spring wheat stored at different moisture contents in an atmosphere of air (open circles) or nitrogen (solid circles) at 20°C.

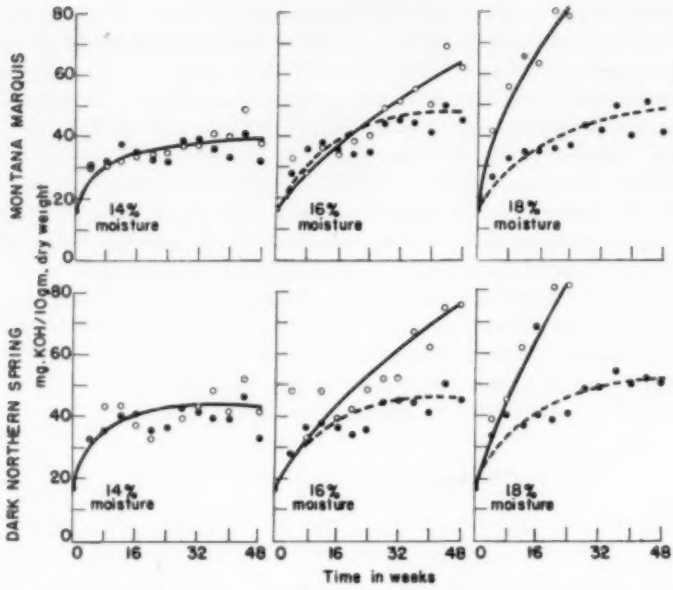

Fig. 6. Changes in fat acidity of Marquis and a composite sample of dark northern spring wheat stored at different moisture contents in an atmosphere of air (open circles) or nitrogen (solid circles) at 20°C.

increased rapidly with increasing time and moisture content in the grain stored in air.

Reducing and nonreducing sugars: In all cases, reducing sugars increased and nonreducing sugars decreased upon storage (Table VI). At 16% moisture, the changes which occurred in the two atmospheres were quite similar. This was also the case for the reducing sugars at the end of 24 weeks at 18% moisture. Nonreducing sugars decreased considerably faster in the air-stored samples at 16 and 18% than was the case with the samples stored in nitrogen.

Mold count and carbon dioxide production: In the grain stored at 16 and 18% moisture in air, molds increased rapidly and large amounts of carbon dioxide were produced (Table VII). In the grain stored in nitrogen, molds did not increase, and only a negligible amount of carbon dioxide was produced. The sample of No. 2 composite dark northern spring wheat, for example, at 16% moisture in air produced 84 times as much carbon dioxide as that stored in nitrogen.

Germ damage: No germ damage developed at 14% moisture. "Sick" wheat developed very slowly in the nitrogen-stored samples at 18%

TABLE VI

EFFECT OF ATMOSPHERE AND MOISTURE CONTENT ON REDUCING AND NONREDUCING SUGARS IN WHEATS STORED AT 20°C.

Storage Time	Moisture Content, Marquis (Dry-Matter Basis)			Moisture Content, Dark Northern Spring (Dry-Matter Basis)		
	14%	16%	18%	14%	16%	18%
weeks	*mg/10 g*	*mg/10 g*	*mg/10 g*	*mg/10 g*	*mg/10 g*	*mg/10 g*
	Reducing sugars in air[a]					
24	30	42	54	32	43	64
48	34	52	..	34	53	..
	Reducing sugars in nitrogen[a]					
24	30	38	50	30	40	64
48	34	51	97	32	60	113
	Nonreducing sugars in air[b]					
24	205	190	105	226	174	101
48	201	155	...	198	141	...
	Nonreducing sugars in nitrogen[b]					
24	210	189	152	208	205	164
48	208	197	144	206	189	137

[a] Initial values: Marquis, 37 mg. per 10 g. wheat; dark northern spring, 25 mg. per 10 g. wheat.
[b] Initial values: Marquis, 237 mg. per 10 g. wheat; dark northern spring, 230 mg. per 10 g. wheat.

moisture and hardly at all in those at 16%; darkened germs were present only in the last sample of the No. 2 composite dark northern spring wheat stored at 16% moisture. In the air-stored samples, however, the appearance of "sick" wheat was very rapid and was complete in both wheats held at 18% moisture after 16 weeks. With the exception of the Marquis wheat stored in nitrogen at 16% moisture, all samples contained essentially 100% damaged germs after 48 weeks. (Table VIII.)

TABLE VII

EFFECT OF MOISTURE CONTENT AND STORAGE ATMOSPHERE ON MOLD GROWTH AND CARBON DIOXIDE PRODUCTION IN WHEATS STORED AT 20°C. FOR 24 WEEKS

Wheat Sample	Moisture	Mold Growth[a]		Carbon Dioxide Production[b]	
		Air	Nitrogen	Air	Nitrogen
	%				
Montana Marquis	14	0	3	0.21	0.12
Montana Marquis	16	30	0.5	18.0	0.37
Montana Marquis	18	7900	0	89.2	3.56
Dark northern spring	14	1.5	1.5	0.17	0.04
Dark northern spring	16	2330	2.5	25.2	0.30
Dark northern spring	18	45,500	0.5	127.0	2.82

[a] Mold counts expressed as thousands per g. wheat.
[b] Average weekly carbon dioxide production as mg. CO_2 per 100 g. wheat (dry-matter basis).

TABLE VIII

EFFECT OF MOISTURE CONTENT AND STORAGE ATMOSPHERE ON THE DEVELOPMENT OF GERM DAMAGE IN WHEAT AT 20°C.

Time	Germ Damage, Percent							
	Marquis				Dark Northern Spring			
	Moisture Content of Air		Moisture Content of Nitrogen		Moisture Content of Air		Moisture Content of Nitrogen	
	16%	18%	16%	18%	16%	18%	16%	18%
weeks								
0	0	0	0	0	0	0	0	0
8	0	20	0	0	0	60	0	0
16	0	100	0	0	0	100	0	5
24	0	100	0	0	21	100	0	10
32	5	100	0	10	100	100	0	10
40	30	100	0	26	100	100	0	100
48	95	100	0	95	100	100	95	100

Discussion

At 30°C. the onset of deterioration, as measured by decrease in germination and by increase in germ damage, free fat acidity, reducing sugars, carbon dioxide production, and mold count, was delayed somewhat by storage in nitrogen as compared with storage in air. Once deterioration had begun in the grain stored in nitrogen, it progressed about as rapidly as in the grain stored in air. Storage in nitrogen prevented mold growth, but did not prevent decreases in nonreducing sugars, increases in reducing sugars, or the development of germ damage. These changes in the grain stored in nitrogen must therefore be attributed to the hydrolytic activity of the wheat enzymes which catalyze the reactions which are involved. In the samples stored in air, fungi developed and the decreases in nonreducing sugars occurred at a faster rate than in those stored in nitrogen. This is attributed to the combined effect of the enzymes present in the wheat and those elaborated by the molds. In contrast, the reducing sugars increased at a slower rate in the samples stored in air; presumably because the molds metabolize these sugars. This is indicated by the greater amounts of carbon dioxide produced by the air-stored samples. Some carbon dioxide, however, was produced in the nitrogen atmosphere, establishing that fermentation was taking place; this is further indicated by the sour, somewhat alcoholic odors which developed in the nitrogen-stored wheat.

The liberation of fatty acids appears also to be due to both wheat and fungal lipases. At 30°C. and moisture contents below 15%, the rather slow increases in fat acidity are probably largely due to wheat lipases. At higher moisture contents where molds proliferated rapidly,

lipase produced by them would probably account for a large percentage of the fatty acids liberated.

At 20°C., deterioration as measured by the criteria listed above was much slower in the samples stored in nitrogen than in those stored in air. Germination remained high (approximately 80%) for 16 weeks even at 18% moisture, and germ damage developed much more slowly than in the samples stored in air. At 16% moisture, the No. 2 composite dark northern spring wheat stored in nitrogen was free of germ damage after 40 weeks, and the Marquis after 48 weeks. The much slower rates of biological change in the nitrogen-stored samples, as compared with those observed at 30°C., indicate that seed metabolism is greatly reduced by a lowering of the temperature.

Results of the present tests suggest that hermetic storage of grain probably would have little effect in prolonging storage life at temperatures above 20°C. If, however, the deteriorative processes continue to slow down at temperatures below 20°C., as they did between 30° and 20°C., moist grain might be kept in hermetic storage for some time without obvious damage. The Marquis wheat was kept for almost a year in nitrogen, at 16% moisture, without development of "sick" wheat, whereas the same grain in air developed 95% germ damage in the same time. The results of laboratory tests such as these cannot be translated directly in commercial practice, of course, since many complications are involved. In all tests the No. 2 composite dark northern spring wheat deteriorated, by all criteria used to judge deterioration, more rapidly than did the very high quality Marquis. Many parcels of commercial wheat that grade No. 2 very probably would not be of as high a quality as the No. 2 composite here used; wheat that has undergone some invasion by storage molds and which already has decreased somewhat in germination and is on the verge of developing germ damage would likely deteriorate faster than the samples in these tests. Also, in these tests the moisture content of the grain was maintained at a constant and uniform level throughout. In large bulks of grain stored in commercial bins the moisture content often varies over a range of several percent. Where a temperature of 20°C. or below could be maintained and the moisture content does not exceed 16%, it might be possible to store wheat for considerable periods in inert gas without obvious damage. Tests with larger bulks, and under conditions that prevail in practice, are necessary to determine the feasibility of such storage.

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THE AMPEROMETRIC TITRATION OF THIOL GROUPS IN FLOUR AND GLUTEN[1]

A. H. Bloksma

ABSTRACT

The results of amperometric titrations of thiol groups in flour with mercury(II) nitrate were between 0.36 and 0.45 thiol groups per 1,000 nitrogen atoms, and depended little upon the medium. Titrations with silver nitrate in ammonia buffers yielded 0.9 and 0.6 –SH per 1,000 N at pH 8 and at pH 10, respectively. By means of titrations with silver nitrate in tris-(hydroxymethyl) aminomethane buffer, 0.8 –SH per 1,000 N were found at pH 8, and smaller amounts in acid or more strongly alkaline solutions; the results were generally lower than in ammonia buffers of the same pH. Using silver nitrate as reagent, the addition of ethylene diamino tetra-acetic acid increased the results by about 17% in weakly alkaline ammonia buffers.

A slow consecutive reaction of silver ions with flour or gluten coincides with low titration results. Therefore this reaction is ascribed to thiol groups, the reactivity of which is hampered by an unfavorable medium. These observations, together with experiments with para-chloromercury(II) benzoic acid, support the assumption of the specificity of the titration.

After completion of a number of amperometric titrations of thiol groups in wheat flour and gluten (3), the author became acquainted with the results of similar experiments by Sokol, Mecham, and Pence (21) and by Matsumoto and Hlynka (16). Using a different titration medium, these investigators obtained higher results than did the present author. The experiments described in Part I of this paper aimed to trace the cause of these variations.

Part II deals with the possibility of a side reaction that might interfere with the specificity of the titrations of thiol groups with silver or mercury(II) nitrate.[2] This possibility was checked by three types of experiments, namely: (a) titrations after the addition of the thiol blocking reagent para-chloromercury(II) benzoic acid; (b) titrations in which an excess of reagent was allowed to react with the sample for a certain time; and (c) a study of the decrease in diffusion current with time after completion of the titration.

Materials and Methods

A flour of 71% extraction was milled on a Bühler laboratory mill from the wheat variety Carsten's VI. This untreated flour ($N \times 5.7 = 7.0\%$; ash 0.34%, both on a 14% moisture basis) was used for all titra-

[1] Manuscript received September 10, 1958. Contribution from the Institute for Cereals, Flour and Bread T.N.O., Wageningen, The Netherlands.

[2] In accordance with the recommendations of the Inorganic Nomenclature Committee of the I.U.P.A.C., Roman figures are used to denote the valency of elements in their compounds.

tions with flour. Gluten A ($N \times 5.7 = 86.0\%$ on dry basis) was separated from this flour and lyophilized as described previously (3). Gluten B ($N \times 5.7 = 76.3\%$ on dry basis) was separated from a flour milled from a blend of Dutch wheat varieties; in this case the removal of starch residues by centrifugation of the acetic acid dispersion was omitted.

Thiol groups were titrated under oxygen-free nitrogen with 0.001*M* silver nitrate or a solution containing 0.001*M* mercury(II) acetate and 0.0015*M* nitric acid (henceforth referred to as mercury(II) nitrate solution). When an excess of silver nitrate was to be added (see Part II), a 0.005*M* solution was used in order to limit the increase of volume. Every 40 seconds a 0.4-ml. portion of reagent was added.

The end point of the titrations was detected amperometrically by means of a stationary platinum wire electrode in a titration vessel with a magnetic stirrer, shown previously (3). The potential of the platinum electrode is given in Table I. The electrode was cleaned regularly, and at least when one reagent was replaced by the other, by anodic oxidation or with warm concentrated nitric acid. In most cases the temperature of the titration vessel was controlled thermostatically.

TABLE I

POTENTIAL OF THE PLATINUM ELECTRODE

Reagent Ion	Medium	Code of Table II	Potential vs. SCE [a] Volts
Mercury	All	A–H	−0.15
Silver	Ammonia buffers	I–O	−0.30
Silver	Acid and weakly alkaline tris buffers	P and Q	−0.10
Silver	Strongly alkaline tris buffers	R	−0.15

[a] Saturated calomel electrode.

The composition of the solvents is given in Table II. Concentrations are reported in molarity. Before addition of the samples, oxygen was removed from the solvents by bubbling nitrogen through.

For most titrations 2 to 4 g. of flour or 0.25 to 1 g. of gluten was dispersed in 100 ml. solvent; except that for the titrations referred to in Fig. 2 and in the first lines of Table IV, 50 ml. were used. For titrations in the solvents A, B, I, and K, containing less than 6*M* urea or acetamide, the flour was mixed in a mortar, with part of the solvent water, into a thick slurry that was added to the remainder of the solvent. When the ethanol containing solvent J was used, the flour was added directly to the solvent. For titrations in the remaining solvents, the flour was dispersed in the electrolyte solutions prior to the addition of urea. Gluten was added to the solvents as an acetic acid disper-

sion, except for the titrations of Fig. 2 and of the second line of Table IV.

Variations in this titration method, used for a check of the specificity of the reaction, are described in Part II of this paper.

Water was redistilled, the second time in a Pyrex glass apparatus. Ethanol was distilled from silver nitrate. Potassium chloride was recrystallized twice from water; to the first solution 80 micromoles mercury(II) nitrate per kg. potassium chloride were added. Tris-(hydroxymethyl) aminomethane, hereafter indicated by "tris" (systematic name 2–amino–2–hydroxymethyl–1,3-propanediol), was Sigma 121 (from Sigma Chem. Co., St. Louis, Mo.); para-chloromercury(II) benzoic acid, hereafter indicated by "PCMB," was from Hopkin & Williams, Chadwell Heath, Essex, England. Ethylene diamino tetraacetic acid, hereafter indicated by "EDTA" (systematic name (ethylenedinitrilo)–tetraacetic acid), was from unknown origin. The other reagents were of analytical reagent-grade quality, except urea (puriss. cryst., Merck, Darmstadt, W.–Germany), acetamide (Merck), and ammonium chloride (puriss., satisfying the requirements of Ph.Ned., Ed. V). The average of blank titrations was 5×10^{-7} moles per liter; results were corrected for blank values.

Results and Discussion

Part I. The Influence of the Medium

Results are summarized in Table II and Fig. 1.

The media A, I, and J were used by the author previously (3); Sokol, Mecham, and Pence (21) and Matsumoto and Hlynka (16) used medium P. Table II shows that application of these four media to the same flour furnished results with medium P that are higher than with the media A, I, and J. Obviously the variations mentioned above have not been due to differences between the flours investigated; they must be ascribed to differences between the media applied. These differences may refer to the type and amount of the denaturing agent, the type and pH of the buffer, the presence of EDTA, the temperature, and the reagent. Moreover, the ionic strength of the solution may vary, but, as a consequence of the technique applied, must be high. The following paragraphs aim to determine which of the above factors are the most important.

All titrations with mercury(II) nitrate as reagent (A through H in Table II) yielded results that did not scatter nearly as widely as the results of titrations with silver nitrate under various conditions. Assuming that one mercury atom reacts with one thiol group, the average result of all titrations with mercury(II) nitrate is 0.41 –SH

TABLE II

RESULTS OF TITRATIONS OF FLOUR IN DIFFERENT MEDIA

MEDIUM				–SH PER 1,000 N		
Code and Composition	pH	Temp.	REAGENT ION	Mean Result	No. of Titrations	Stand. Dev.
		°C				
A—3.4*M* urea/0.4*M* KCl/0.04*M* acetic acid	4.2–4.4	20	mercury	0.39	16	0.03
B—3.4*M* urea/0.4*M* KCl/0.04*M* acetic acid/8.3×10^{-4}*M* EDTA	4.1	20	mercury	0.36	3	0.01
C—8*M* urea/0.4*M* KCl/0.04*M* acetic acid/3×10^{-4}*M* EDTA	4.5	2	mercury	0.45	13	0.06
D—8*M* urea/0.4*M* KCl	6.8	2	mercury	0.45	7	0.03
E—8*M* urea/0.4*M* NH_4Cl/0.04*M* acetic acid	4.5	2	mercury	0.40	4	0.01
F—8*M* urea/0.4*M* NH_4Cl	6.4–6.8	2	mercury	0.40	4	0.006
G—8*M* urea/ 0.4*M* NH_4Cl/$2.5–4 \times 10^{-3}$*M* NH_4OH	8.0–8.3	2	mercury	0.37	3	0.02
H—8*M* urea/1*M* NH_4NO_3/5×10^{-3}*M* NH_4OH	7.7	2[a]	mercury	0.40	3	0.01
I—3.4 acetamide/0.05*M* NH_4NO_3/0.25*M* NH_4OH	10.2	20	silver	0.44	8	0.04
J—48% ethanol/3.4*M* acetamide/0.05*M* NH_4NO_3/0.25*M* NH_4OH	10.4	20	silver	0.44	7	0.03
K—3.4*M* urea/0.05*M* NH_4NO_3/0.25*M* NH_4OH/EDTA[b]	10.4	2	silver	0.63	5[c]	0.03
L—8*M* urea/0.05*M* NH_4NO_3/0.25*M* NH_4OH/8.3×10^{-4}*M* EDTA	10.2–10.4	2	silver	0.56	4	0.02
M—8*M* urea[d]/1*M* NH_4NO_3/5×10^{-3}*M* NH_4OH/8.3×10^{-4}*M* EDTA	7.9– 8.1	2	silver	0.94	54[d]	0.10
N—8*M* urea/1*M* NH_4NO_3/5×10^{-3}*M* NH_4OH	8.1	2	silver	0.79	6	0.04
O—8*M* urea/0.4*M* NH_4Cl/$0.5–4 \times 10^{-3}$*M* NH_4OH	7.5– 8.3	2	silver	0.65	7	0.03
P—8*M* urea/0.01*M* KCl[e]/0.133*M* tris/0.113*M* HNO_3/3×10^{-4}*M* EDTA	7.9– 8.1	2	silver	0.77	15	0.06
Q—8*M* urea/0.01*M* KCl/0.133*M* tris/0.45–0.17*M* HNO_3/3×10^{-4}*M* EDTA	3.3– 4.4	2	silver	0.55	4	0.02
R—8*M* urea/0.01*M* KCl/0.133*M* tris/0.0085*M* HNO_3/8.3×10^{-4}*M* EDTA	10.2	2	silver	0.48	6	0.02

[a] One titration at 20°C. also yielded 0.40 –SH per 1000 N.
[b] In two cases 8.3×10^{-4} *M* EDTA was added without effect on the results.
[c] Identical with the titrations on the second line of Table III.
[d] The titrations of the first line of Table III are included. See also footnote b, Table III.
[e] In two cases potassium nitrate was substituted for potassium chloride without effect on the results.

per 1,000 N. Results obtained with silver nitrate as reagent in weakly alkaline solutions are about twice as high (M through P; 0.65–0.94 –SH per 1,000 N); this raises some doubt as to the assumed 1:1 mole ratio of the reaction. The addition of EDTA to an acid solution at 20°C. caused a small decrease in the results (A and B). The increase in the results caused by the simultaneous increase in the urea concentration and the decrease in temperature of the acid solutions (B and C) is the largest difference found in the series A through H. Matsumoto and Shimoda found an apparent increase of the thiol content of 10% when the pH was raised from 4.4 to 8.7 (17). In series C-D and E-F-G this result was not confirmed. From the ionization constants of EDTA (18), and the dissociation constants of the mercury-EDTA complex (10) and of the mercury-chloride complexes (8), it is estimated that in a 0.4*M* chloride solution the latter complexes are stable at pH below 5.2; above this pH most of the mercury will be bound to EDTA. In

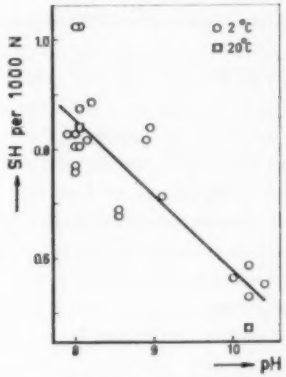

Fig. 1. Titrations with silver nitrate of 3 g. flour dispersed in 100 ml. ammonia buffers of different pH values. The solutions were 8*M* in urea and 3.0 or 8.3×10^{-4}*M* in EDTA.

fact in neutral or alkaline solutions containing EDTA, added mercury was bound in a form that was not reduced at −0.15 V. Therefore EDTA was not applied in these solutions for titrations with mercury (II) nitrate.

The results of titrations with silver nitrate in ammonia buffers (I through O) show that, contrary to previous results (3), the addition of ethanol to a strongly alkaline ammonia buffer with acetamide did not increase the titration result (I and J). The urea concentration had little influence (K and L). The addition of EDTA seems to increase the results, at least in weakly alkaline solutions (M and N; compare

also K). Neglecting the difference between acetamide and urea, one may conclude from a comparison of I and K that the results are higher at 2° than at 20°C. It has not been established by the author that this also holds for weakly alkaline solutions (see Fig. 1). Obviously, the urea concentration, the temperature, and EDTA affect the results in ammonia buffer in the same way as Sokol *et al.* (21) found them to affect the results in tris buffer. The results increase strongly with a decrease of the pH from 10 to 8 (L and M and Fig. 1). The buffer anion probably affects the results also (N and O). A similar observation was made by Simpson and Saroff using methylmercury (II) iodide as reagent; the apparent thiol content of serum albumin decreased more quickly in dilute hydrochloric acid than in nitric acid (19). A comparison of G and O and of H and N shows that in these identical media the results with silver nitrate are higher than with mercury(II) nitrate; in more strongly alkaline solutions the same results were obtained with both reagents (3).

The results of titrations with silver nitrate in tris buffer were lower than in ammonium nitrate buffer of the same pH (M and P). From the ionization constant of tris (9) and the dissociation constants of the silver-tris complex (1), one can conclude that in a 0.01*M* chloride solution, tris cannot prevent the precipitation of silver chloride at a pH below 7. This was confirmed by the shape of the titration curves of blank titrations in acid solutions, and by the appearance of a visible precipitate at higher silver concentrations. Nevertheless, titrations with flour in acid solutions seemed successful (Q). In spite of a possible precipitation of silver chloride, results were low. Therefore fewer thiol groups have reacted with the reagent (in this case uncomplexed silver ions) than in weakly alkaline solutions. In strongly alkaline tris buffer the results were also low (R).

The most striking feature of the results is their dependence upon the pH for the titrations with silver nitrate in ammonia as well as in tris buffers. This dependence is exactly the reverse of that which would be expected if the reactivity of thiol groups in flour proteins were determined completely by their accessibility; at a pH far from the isoelectric point the peptide chain will be more stretched, and consequently, reactive groups will be more easily accessible. On the other hand, titration results with mercury(II) nitrate do not show any effect of the pH.

The influence of most of the factors enumerated in the first paragraph of Part I might be caused by the effect of the solvent upon the state of the proteins in the dispersion. This does not hold for the influence of the reagent. The lack of relation between the results with

mercury(II) and results with silver nitrate, respectively, indicates that other factors than solvent effects play a role. The author is not able to explain the influence of the reagent by means of the sizes of the hydrated or complexed ions.

The present results involve a warning as to the interpretation of amperometric titrations of thiol groups in proteins. The agreement between titrations with different reagents, and at widely deviating pH values, reported previously (3), now proves to be accidental. The higher results, obtained by Sokol, Mecham, and Pence (21) and by Matsumoto and Hlynka (16), are not the highest possible. Even in the case of a simple compound such as cysteine and using the same medium different workers obtained deviating results (14,20). In connection with conflicting results of amperometric titrations of thiol groups in hemoglobin, Hommes *et al.* (11,12) believe that the platinum electrode should be covered with a layer of mercury in order to obtain correct results from titration with silver nitrate. At all events, the condition of the electrode surface must be regarded (14).

Part II. The Specificity of the Titration

An obvious way to test the specificity of the titrations with silver and mercury(II) nitrate is to study the effect on the titration results of a recognized thiol-blocking reagent such as para-chloromercury(II) benzoic acid. Figure 2 shows that in a series of titrations of gluten with silver nitrate the results after addition of variable amounts of

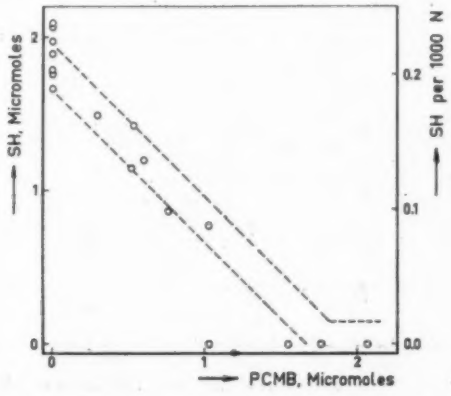

Fig. 2. Titrations with silver nitrate of 1 g. gluten B in 50 ml. solvent J (see Table II) after addition of different amounts of para-chloromercury(II) benzoic acid (PCMB). The dotted lines have the theoretical slope; their distance is two times the standard deviation of the results reduced to zero addition of PCMB.

PCMB decreased in accordance with a stoichiometric reaction. This observation confirms that both silver nitrate and PCMB react with the same functional groups, presumably thiol groups. To check further the specificity of the titration with silver nitrate, two other methods were used.

The results of the titrations will depend on the time of contact between the reagent and the sample, if the reagent reacts slowly either with thiol groups that are not easily accessible, or with other than thiol groups. Both cases have been described in the literature. Kolthoff *et al.* found slowly reacting thiol groups in denatured serum albumin (13). Silver (4,5,6) or mercury(II) salts (2,7,15) can react with small disulfides or accelerate their hydrolysis. Since flour and gluten contain large amounts of disulfide groups as compared with thiol groups (3), a reaction between the reagent and these disulfide groups must be envisaged; because of the previous results (3) this reaction cannot be a fast one.

One of the procedures applied to study the effect of the time of contact upon the titration results was that of Kolthoff *et al.* (13). An excess of reagent was added quickly, and after a reaction time of 10, 20, or 30 minutes the titration was continued in the usual way. The consumption of reagent was determined by extrapolation of the ascending

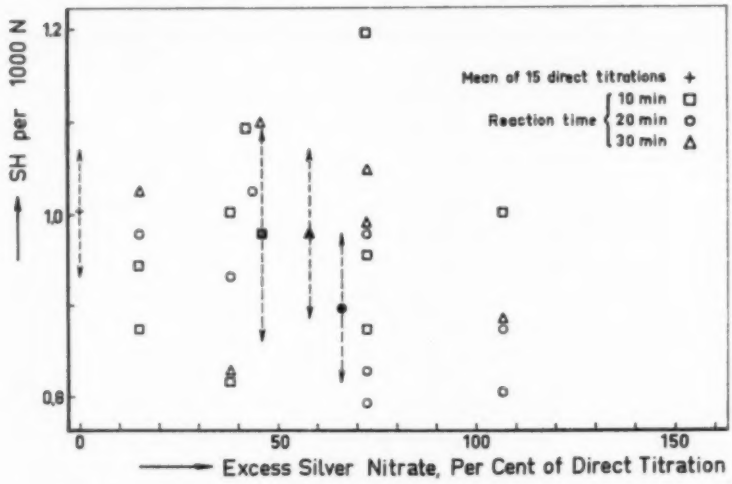

Fig. 3. Titrations with silver nitrate of 2 to 3 g. flour in 100 ml. solvent M (see Table II). The open points indicate individual observations. Filled-in points are mean values; the arrows indicate the square root of the variance of the results.

limb of the titration curve. During the reaction time the electrical leads were disconnected. The results (Fig. 3 and Table III) do not indicate a slow side reaction except in one case. The titration of gluten in a strongly alkaline solution at 20°C. (last line of Table III) is the only case in which the results of titrations with reaction times are significantly higher than those of direct titrations.

A more sensitive procedure is to study the decrease of the diffusion current with time after completion of a titration. It is assumed that the diffusion current, i, is proportional to the concentration, c, of the reagent. Their constant ratio is called $a \equiv i/c$; a can be determined from the slope of the ascending limb of the titration curve. Since the electrical current is caused by a discharge and deposition of reagent ions, the decrease of the concentration is described by

$$-\frac{1}{a} \cdot \frac{di}{dt} = -\frac{dc}{dt} = \frac{i}{zFv}$$

in which t is time, z is the valency of the ions, F the Faraday (9.65×10^4 Coulomb per gram-equivalent), and v the volume of the solution. Integration of this equation yields:

$$\ln i = \ln i_0 - \frac{at}{zFv} \qquad (1),$$

in which i_0 is the current at zero time. If the disappearance of reagent ions is caused exclusively by processes the rate of which is proportional to the reagent ion concentration, then the experimentally found relation will be:

$$\ln i = \ln i_0 - bt \qquad (2).$$

TABLE III

INFLUENCE OF REACTION TIME ON THE RESULTS OF TITRATIONS WITH SILVER NITRATE OF FLOUR AND OF DRY GLUTEN FROM THIS FLOUR[a]

Sample	Medium (Code of Table II)	Thiol Groups per 1,000 Nitrogen Atoms					
		Direct Titrations			Titrations with Reaction Time		
		Mean Result	No. of Titrations	Stand. Dev.	Mean Result	No. of Titrations	Stand. Dev.
Flour	M[b]	1.00	15	0.07	0.95	23	0.10
	K[c]	0.64	2	0.02	0.61	3	0.03
Gluten A	M	0.33	6	0.02	0.35	6	0.04
	K[d]	0.145	5	0.006	0.199	4	0.014

[a] The first line of the table refers to the same experiments as does Fig. 3. With the other experiments the excess of silver nitrate added was 25 to 80% and reaction times were 10, 20, or 30 minutes.
[b] In some cases the urea concentration was decreased to 6*M* without effect on the results.
[c] In some cases EDTA was added up to a concentration of 8.3×10^{-4}*M* without effect on the results.
[d] The temperature was 20°C. instead of 2°C.

In this equation b is a constant that is equal to or greater than a/zFv. The difference $b - a/zFv$ is a measure of the rate of disappearance of reagent ions in other ways than by deposition on the electrode; it should be considered as the rate constant of a first-order reaction. The author preferred the determination of this rate by means of a subtraction to connecting the electrical leads intermittently, because the current often is irregular during starting periods.

TABLE IV

RATE OF SILVER CONSUMPTION AFTER COMPLETION OF THE TITRATION[a]

MEDIUM (CODE OF TABLE II)	SAMPLE[b]	$\frac{v}{l}$	a A.l/mole	$b \times 10^6$ sec^{-1}	$(b - a/zFv) \times 10^6$ sec^{-1}
I	blank	0.052	1.51	2.74	−0.2
		0.052	1.60	3.24	+0.1
	250 mg. gluten B	0.052	0.95	3.87	2.0
	4 g. flour	0.055	0.78	4.37	2.9
J	4 g. flour	0.055	0.65	2.16	0.9
K[c]	blank	0.101	2.80	3.10	0.2
	716 mg. gluten A	0.103	1.74	3.54	1.8
		0.103	1.55	3.85	2.3
M	blank	0.102	1.28	1.32	0.01
	2 g. flour	0.103	0.73	0.71	−0.02
	2.5 g. flour	0.103	0.66	1.10	0.44
		0.104	0.67	0.70	0.03

[a] For meaning of symbols, see text.
[b] In this column the weight of gluten refers to 5.7 times the amount of nitrogen; the weight of flour refers to the flour as-is.
[c] The temperature was 20° instead of 2°C.

In fact, the decrease of the current with time could be described by equation 2. Table IV shows the values of a and b, calculated from the slope of the ascending limb of the titration curve and from the decrease of the current with time, respectively. In the media I and J, and in K at 20°C., the rate of disappearance of silver ions by other causes than the electrical current is of the order of $2 \times 10^{-4}\,sec^{-1}$, provided that flour or gluten is present. This means that an excess of 60% is reduced to 47% after 20 minutes' reaction time; the result of such a titration would have been 13% higher than that of the corresponding direct titration. It is doubtful whether this difference would have been found by means of the technique of Kolthoff *et al.* (13).

The data in Tables III and IV indicate a slow consecutive reaction in strongly alkaline media at room temperature. Under these conditions the titration results are low. Therefore it is highly probable that this consecutive reaction is due to thiol groups, the reactivity of which is hampered in these unfavorable media, and which consequently did not react with silver nitrate before the end of a quick, direct titration.

The coincidence of low titration results and a consecutive reaction is not explained if this reaction is due to other than thiol groups.

The experiments of Part II support the assumption that titrations with silver nitrate only establish thiol groups, though not necessarily all of them.

Acknowledgment

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STALING STUDIES OF BREAD MADE WITH FLOUR FRACTIONS

V. Effect of a Heat-Stable Amylase and a Cross-Linked Starch[1]

W. G. BECHTEL

ABSTRACT

Bread was made of gluten and wheat starch or a mixture of wheat starch and a cross-linked starch derivative which contained up to 80% of the latter. Part of the bread was supplemented with bacterial alpha-amylase at 4 and at 24 SKB units per lb. Freshly baked bread was increasingly stale and firm as the proportion of cross-linked starch was increased. During a 6-day storage period, bread staled and firmed less with increasing levels of bacterial alpha-amylase. Changes in starch are shown to be of major importance in the staling and firming of bread.

Many studies of bread staling which have been made during the past 70 years have attributed the staling of bread to changes in the properties of starch. Most of the evidence has been indirect, depending on inferences drawn from separate studies of the aging of starch pastes and the changes in one or more of the physical properties of bread crumb during storage. Summaries of these studies and references to the original publications are to be found in reviews by Bice and Geddes (8), MacMasters and Baird (10), and Bechtel (2). Interpretation of much of this research is difficult because bread freshness is now recognized to be a matter of consumer judgment. In a critical review of staling methods, Bice and Geddes (7) concluded that sensory tests made by a panel of experts under statistical control constitute the fundamental method for determining bread freshness, and that other

[1] Manuscript received September 5, 1958. The research on which this paper is based was conducted by the American Institute of Baking under contract with the U. S. Department of Agriculture under authority of the Research and Marketing Act of 1946. This contract was supervised by the Northern Utilization Research and Development Division of the Agricultural Research Service. Paper was presented at the 43rd annual meeting, Cincinnati, Ohio, April 1958.

methods are valid only if they agree with sensory tests. In recent years some bread staling research has been conducted which meets this criterion. Among such studies are those by Bechtel and Meisner (5,6) and Prentice, Cuendet, and Geddes (13).

The present study was undertaken to obtain direct evidence on the relationship of changes in starch to bread staling. *Staling* is defined as meaning all of the changes which occur in bread during storage which make it less palatable, except those due to the action of spoilage organisms. The term *staling* will be used only to describe results obtained by sensory panel tests.

Materials and Methods

Bread. Bread was made from starch and gluten by the general method described by Bechtel and Meisner (3). A commercial baker's patent spring wheat flour, which was bleached and bromated but not enriched, was used as the source of gluten and wheat starch. Its protein content was 12.6%, and ash was 0.47% at 14% moisture. All bread was made with starch and gluten calculated to produce a "flour" of 12.9% protein at 14% moisture.

To study the effect of a swelling-resistant starch, portions of wheat starch were replaced by Vulca 100,[2] a cross-linked derivative of ungelatinized corn starch composed of starch granules which swell only slightly when cooked with water. After being cooked to 90°C., its susceptibility to the action of amylolytic enzymes was found to be one-seventh that of wheat starch similarly cooked. A heat-stable bacterial alpha-amylase supplement[3] was used because of its effectiveness in minimizing the rate of bread firming (11). The thermal stability of bacterial alpha-amylase has been well established (11,14). The product used in these studies retained 35% of its original activity after it was heated to 90°C.

It was necessary to add increasing amounts of sugar to the sponge as the proportion of cross-linked starch was increased in order to obtain good fermentation. Since this product had a higher absorption than wheat starch, the breads were made in two series. The first, using a lower absorption, consisted of breads made with wheat starch and with 20% and 40% of the wheat starch replaced by cross-linked starch. The second consisted of breads with 40, 60, and 80% of the wheat starch replaced by cross-linked starch. Bread with 40% cross-linked starch and 60% wheat starch was made at both absorptions so that there would be three breads in each series. The baking procedure was as follows:

[2] National Starch Products, Inc., New York, N. Y.
[3] Enzyme Preparation No. 1, Wallerstein Laboratories, New York, N. Y.

Ingredients	*Sponge*	*Dough*
	g	*g*
Gluten (a)	171	171
Starch	255	255
Water	(b)	(b)
Yeast, compressed	13	..
Yeast food, Arkady	3.25	..
Bacterial alpha-amylase	(c)	..
Sugar, sucrose	(d)	39
Salt	..	13
Nonfat dry milk	..	26
Lard	..	26

Mixer: Hobart A-120 with McDuffee bowl and fork. Mixing time for both sponge and dough was 3 minutes at speed 1 followed by 3 minutes at speed 2. Sponge fermentation time, 4 hours. No floor time. Intermediate proof 12 minutes. Proofed to height, ¼ in. above pan. Loaves scaled at 18½ oz. Baked 21 minutes at 450°F.

NOTES: (a) Gluten was frozen without drying and stored until used. Its average composition was water 65%; protein, dry basis, 70%. The amounts of gluten and starch were calculated to give 650 g. flour at 14% moisture.

(b) For breads in the series with wheat starch, and with 20 and 40% of the wheat starch replaced by cross-linked starch, 125 ml. water were used in the sponge and 100 ml. in the dough. For breads in the series with 40, 60, and 80% of the wheat starch replaced, 140 ml. water were used in the sponge and 120 ml. in the dough.

(c) The amount was calculated to give either 4 or 24 SKB units alpha-amylase activity per pound of bread, based on enzyme activity before baking.

(d) Cross-linked starch, %	20	40	60	80
Sugar added to sponge, *g.*	3.0	5.5	8.5	11.0

Quality Score. Bread was evaluated for specific volume by displacement of rapeseed. It was scored also for break and shred, loaf appearance, crust color, grain, texture, crumb color, and flavor. The score is a weighted average of the results expressed on the scale: *excellent*, *very good*, *good*, *fair*, and *poor*.

Bread Storage. Bread was wrapped and sealed in waxed paper and stored in a thermostatically controlled cabinet at 75°F. (24°C.).

Staling Tests. These were made by the procedure described by Bechtel and Meisner (4), using a trained, tested panel of 20 members. A symmetrical 6-point rating scale was used, with ratings *very fresh*, *fresh*, *slightly fresh*, *slightly stale*, *stale*, and *very stale*. Panel members were not asked to explain their judgments in terms of specific crumb properties; therefore no information was obtained about the relative importance of changes in firmness, texture, aroma, and flavor. For statistical analysis the rating *very stale* was given the value of 1 and other ratings were given successive integers to 6 for *very fresh*.

Compressibility. Compressibility was measured with the Baker compressimeter (12) using slices of center crumb 1 by 1 by ½ in. and a compression of 2.5 mm. Compressibility is the average of tests of seven alternate slices from one loaf of bread.

Analytical Methods. Methods for alpha-amylase activity and bread

moisture were those of *Cereal Laboratory Methods* (1). Enzyme susceptibility of the starches was determined by cooking 1.0 g. starch in 100 ml. water to 90°C. in the amylograph. The reducing power was then found by digestion with Takadiastase, followed by determination of reducing sugars as maltose. Thermostability of the bacterial alpha-amylase was found by the method of Miller, Johnson, and Palmer (11).

Results

Comparison of Bread Made from Gluten and Wheat Starch with That Made of Flour. From the flour from which the gluten and wheat starch were obtained, bread was made by the conventional sponge and dough process to serve as a standard for evaluating the experimental breads. Ingredient proportions were the same as for the experimental loaves. Specific volumes of the different lots were between 9.5 and 10.0 cu. in. per oz. Quality scores were from *excellent* to *very good.* Bread made of gluten and wheat starch ranged in specific volume from 9.4 to 10.0 cu. in. per oz. Scores of the different lots were all *very good.*

As shown in Table I, the initial freshness of the bread made from gluten and wheat starch was the same, within experimental error, as that of the bread made of flour at all levels of bacterial amylase. Final freshness ratings of these breads, after 140 hours, were in good agreement when no bacterial alpha-amylase was added, and when the level was 4 SKB units per lb. When bacterial alpha-amylase was added at the level of 24 SKB units per lb., agreement of freshness ratings was good through 44 hours, at which time the final test of bread of flour was made. The crumb of this bread was very gummy when fresh and gumminess increased with storage time. After 44 hours panel members found it too soggy for further tests.

Comparison of the firmness of these breads, Table II, shows reasonably good agreement in their initial firmness and in their firming during the 140-hour storage period. It thus appears that in quality score, in staling, and in firming characteristics bread made from gluten and wheat starch closely approximated that made from flour.

Quality of Bread with Cross-Linked Starch Substituted for Part of the Wheat Starch. The series of breads made with wheat starch only and with 20 or 40% of the wheat starch replaced by cross-linked starch had an average loaf moisture of 35.0%. Specific volumes were between 9.0 and 9.8 cu. in. per oz. The scores were *very good* for breads with 0 or 20% cross-linked starch and *good* for that with 40%. The loaf moisture of the series with 40 to 80% of the wheat starch replaced by cross-linked starch was 36.6%. Specific volumes of these loaves were between 8.0 and 8.9 cu. in. per oz. Quality scores were *good* for bread

with 40% cross-linked starch and *fair* for those with 60 or 80%. While specific volume and quality score decreased with increase in cross-linked starch, within each series the range was not great. The crumb of breads with 24 SKB units of bacterial amylase was gummy, although considerably less gummy than that made with flour. Gumminess decreased with each increment of cross-linked starch.

Initial Freshness of Breads. In general, at each level of bacterial alpha-amylase the initial panel test of breads made from gluten and starch showed a decrease in freshness as the proportion of cross-linked starch was increased. Freshness values at 2 hours in Table I show that the only exception was for breads with 0 and 20% cross-linked starch supplemented with 24 SKB units of bacterial alpha-amylase per lb. These breads were given the same rating of 5.9. Comparing the extremes in each bread series, differences in initial freshness of breads with 0 and 40% cross-linked starch ranged from 1.2 rating scale units for breads with no bacterial alpha-amylase to 0.5 units for those with 24 SKB units per lb. For breads with 40 and 80% cross-linked starch the difference was 2.0 scale units at each level of bacterial alpha-amylase.

With the exception of those which contained only wheat starch, breads of gluten and starch with the same starch composition increased in initial freshness with increase in bacterial alpha-amylase. The initial freshness rating of bread made with wheat starch and no bacterial alpha-amylase was 5.9, while the maximum rating was 6.0. Thus, for this bread it would not be possible to increase the rating appreciably by adding the enzyme. For all others the effect of the change from 0 to 24 SKB units per lb. was to increase the initial freshness approximately the same amount, between 0.7 and 0.9 unit.

Bread Staling. Staling during the 140-hour storage period is given in the extreme right column of Table I. At constant levels of cross-linked starch, bread staled less as the content of bacterial alpha-amylase was increased from 0 or 4 SKB units to 24 SKB units per lb. For example, breads with no cross-linked starch and with 0 or 24 SKB units of bacterial alpha-amylase staled 3.3 and 1.5 scale units, respectively. This is the greatest difference shown. Others ranged from 0.2 units for bread with 20% cross-linked starch to 1.3 units for bread with 40% cross-linked starch in the series from 40 to 80%. In breads with the same level of bacterial alpha-amylase, there was no consistent difference in the amount of staling as the proportion of cross-linked starch was increased.

Initial Crumb Firmness. At each level of bacterial alpha-amylase, the initial crumb firmness became greater with increase in cross-linked

starch. The firmness increases, which are shown in the 2-hour values of Table II and which were caused by the change from 0 to 40% cross-linked starch, were 3.2, 2.3, and 2.2 g. per sq. in. at 0, 4, and 24 SKB units of bacterial alpha-amylase, respectively. Those caused by the change from 40 to 80% cross-linked starch were 2.1, 2.5, and 1.5 g. per sq. in. at 0, 4, and 24 SKB units of bacterial alpha-amylase, respectively. Differences in initial crumb firmness of breads of constant starch composition were very small as the bacterial alpha-amylase was increased from 0 to 24 SKB units per lb.

TABLE I

STALING OF BREAD MADE OF GLUTEN AND STARCH WITH BACTERIAL ALPHA-AMYLASE AND WITH UP TO 80% OF THE WHEAT STARCH REPLACED BY CROSS-LINKED STARCH[a]

VULCA 100 IN STARCH	BREAD FRESHNESS[b] Storage Time, Hours										STALING DURING STORAGE
	2		20		44		92		140		
%	Loaf moisture 35%, no bacterial amylase										
		S.D.		S.D.		S.D.		S.D.		S.D.	
C[c]	5.7	0.56	5.2	0.80	4.9	0.71	4.6	0.91	3.1	1.01	2.6
0	5.9	0.30	4.7	0.78	4.1	0.94	3.1	0.98	2.6	1.09	3.3
20	5.0	0.84	4.2	0.93	3.2	0.87	2.6	0.80	2.4	0.98	2.6
40	4.7	0.78	3.6	0.89	3.2	0.81	2.0	0.72	1.7	0.75	3.0
	Loaf moisture 35%, bacterial amylase 4 SKB units per lb.										
C[c]	5.8	0.51	5.4	0.50	5.0	0.77	4.7	0.93	3.5	0.97	2.3
0	5.9	0.30	4.8	0.95	4.1	0.99	3.1	0.98	3.1	1.13	2.8
20	5.8	0.51	4.6	0.80	3.6	0.91	3.0	0.91	2.5	1.02	3.3
40	5.2	0.93	4.1	0.89	3.6	0.98	2.5	1.01	2.1	0.91	3.1
	Loaf moisture 35%, bacterial amylase 24 SKB units per lb.										
C[c]	5.8	0.51	5.5	0.59	5.5	0.77	[d]		[d]		[d]
0	5.9	0.30	5.3	0.98	4.8	0.87	4.2	1.14	4.4	1.23	1.5
20	5.9	0.22	5.3	0.73	4.5	0.26	4.2	1.08	3.5	1.22	2.4
40	5.4	0.73	5.0	0.80	4.8	0.92	3.9	1.08	3.4	1.18	2.0
	Loaf moisture 36.6%, no bacterial amylase										
40	4.8	0.96	3.9	0.85	3.5	0.84	2.4	0.70	2.4	1.11	2.4
60	3.6	0.92	3.1	0.86	2.2	0.89	2.0	0.72	1.8	0.77	1.8
80	2.8	0.93	2.2	0.70	2.1	0.82	1.7	0.73	1.5	0.74	1.3
	Loaf moisture 36.6%, bacterial amylase 4 SKB units per lb.										
40	5.2	0.78	4.6	0.76	4.1	0.94	3.4	1.06	3.1	0.87	2.1
60	4.0	0.86	3.8	0.98	3.2	1.06	2.5	0.85	2.3	0.93	1.7
80	3.2	0.91	2.9	1.12	2.4	0.94	2.4	1.01	2.1	0.98	1.1
	Loaf moisture 36.6%, bacterial amylase 24 SKB units per lb.										
40	5.6	0.74	5.4	0.55	5.1	1.08	4.8	0.70	4.5	0.85	1.1
60	4.5	0.92	4.6	0.86	4.3	0.97	3.8	1.12	3.6	1.21	0.9
80	3.6	0.97	3.3	1.14	3.2	1.07	3.0	1.14	2.8	1.16	0.8

[a] Values are averages of three panel tests.
[b] Rating scale units: 6, very fresh; 5, fresh; 4, slightly fresh; 3, slightly stale; 2, stale; 1, very stale.
[c] Control; bread made of flour.
[d] These samples were too soggy to test.

Crumb Firming. Increases in crumb firmness during the 140-hour storage period are given in the extreme right column, Table II. In breads of every starch composition, firming became less with increase in bacterial alpha-amylase. Breads with 24 SKB units of bacterial alpha-amylase firmed very little. At equal levels of bacterial alpha-amylase, there was little difference in firming within each bread series as the proportion of cross-linked starch was increased.

TABLE II

FIRMING OF BREAD MADE OF GLUTEN AND STARCH WITH BACTERIAL ALPHA-AMYLASE AND WITH UP TO 80% OF THE WHEAT STARCH REPLACED BY CROSS-LINKED STARCH[a]
(Grams per square inch)

Vulca 100 in Starch	Crumb Firmness										Firming during Storage
	Storage Time, Hours										
	2		20		44		92		140		
%											
	Loaf moisture 35.0%, no bacterial amylase										
		S.D.		S.D.		S.D.		S.D.		S.D.	
C[b]	2.9	0.49	5.0	0.51	6.0	0.87	8.3	1.15	11.7	2.78	8.8
0	2.2	0.52	6.5	0.91	9.8	1.11	11.2	2.28	13.3	2.40	11.1
20	4.3	0.80	9.4	2.30	10.7	2.52	12.9	2.07	16.9	2.57	12.6
40	5.4	0.90	12.4	2.42	14.2	3.12	18.3	2.57	20.3	4.21	14.9
	Loaf moisture 35.0%, bacterial amylase 4 SKB units per lb.										
C[b]	2.9	0.57	4.0	1.01	5.3	0.77	6.7	1.28	8.9	1.30	6.0
0	2.8	0.24	6.5	1.55	9.7	2.78	11.3	1.39	12.5	4.83	9.7
20	3.1	0.32	8.9	1.92	10.2	2.43	11.2	1.91	15.0	3.45	11.9
40	5.1	0.99	10.5	1.55	12.8	2.68	14.3	2.85	15.4	2.76	10.3
	Loaf moisture 35.0%, bacterial amylase 24 SKB units per lb.										
C[b]	3.6	0.44	3.6	0.77	3.2	0.97	3.4	0.70	3.9	1.19	0.3
0	2.9	0.50	4.8	1.32	5.2	1.37	6.1	1.40	6.4	2.22	3.5
20	3.0	0.38	5.4	1.18	5.8	1.46	6.0	2.46	9.4	2.98	6.4
40	5.1	0.79	6.6	1.69	7.1	1.76	8.9	2.22	8.5	2.73	3.4
	Loaf moisture 36.6%, no bacterial amylase										
40	5.2	0.70	8.1	1.55	10.0	1.05	10.4	0.92	10.8	1.11	5.6
60	5.6	0.66	9.8	2.94	11.7	1.05	12.8	2.77	11.8	1.74	6.2
80	7.3	0.96	10.4	1.86	13.5	2.37	12.5	2.61	14.5	1.79	7.5
	Loaf moisture 36.6%, bacterial amylase 4 SKB units per lb.										
40	5.1	0.83	6.6	1.41	7.9	1.09	7.7	1.89	8.5	1.37	3.4
60	5.4	0.69	9.1	3.06	10.2	2.16	10.5	1.76	10.0	1.28	4.6
80	7.6	1.05	10.6	1.80	12.4	1.79	11.6	2.06	10.6	2.07	3.0
	Loaf moisture 36.6%, bacterial amylase 24 SKB units per lb.										
40	5.1	0.32	5.1	1.33	4.2	0.59	4.1	0.63	5.3	0.78	0.2
60	5.3	0.22	6.6	2.04	6.2	0.90	7.1	1.28	5.9	1.10	0.6
80	6.6	2.87	8.9	1.64	9.7	1.45	7.9	2.13	8.1	1.41	1.5

[a] Values are averages of tests of three loaves.
[b] Control; bread made of flour.

Discussion

As shown in the analysis of variance, Table III, the effects of storage time, proportion of cross-linked starch, and level of bacterial alpha-amylase on the staling and firming of bread were significant at the 5% level or below. Most of the first-order interactions were also significant.

TABLE III

ANALYSIS OF VARIANCE OF STALING AND FIRMING OF BREAD DURING A 140-HOUR STORAGE PERIOD

Source of Variation	Degrees of Freedom	Mean Squares			
		Proportion of Vulca 100			
		0 to 40%		40 to 80%	
		Staling	Firming	Staling	Firming
Between storage times	4	31.732**	341.34**	9.381**	77.566*
Between starches	2	5.976*	152.29*	29.601**	164.791**
Between amylase levels	2	16.388**	322.59*	25.332**	204.974**
First-order interactions					
Storage times × starches	8	0.179	4.155	0.343**	8.220**
Storage times × amylase levels	8	0.543**	25.973**	0.476**	16.286**
Starches × amylase levels	4	0.391*	14.274**	0.435**	0.622
Second-order interaction					
Storage times × starches × amylase levels	16	0.0877	1.3642	0.0768	1.9138
Residual	90	0.1420	3.6896	0.1223	1.5660

The principal differences in the experimental breads were the composition of the starch and the level of bacterial alpha-amylase. The proteins were the same in kinds and amounts. To avoid the effect of the necessary change in absorption as the proportion of cross-linked starch was increased, the breads were divided into two series, considered separately, on the basis of moisture content. The only other difference was the increase in sugar with increase in cross-linked starch. The maximum difference in sugar amounted to 0.85% in each series of breads. It has been shown by several investigators that material differences in sugar content do not affect bread firmness or rate of firming (2). Unpublished data from the author's laboratory show that the sensory test panel could not detect a difference of 1% in the sugar content of bread at the level of sugar used in these experiments. Therefore, the effects of the differences in sugar content of these breads appear negligible.

As shown by its low swelling power when cooked with water, the cross-linked starch had much stronger intermolecular bonds than unmodified wheat starch. Zobel and Senti (15), in X-ray diffraction

studies of the starches and certain of the breads used in these experiments, found that freshly prepared gels of cross-linked starch and freshly baked bread which contained this starch gave the "B" pattern shown by Katz (9) to be characteristic of both stale bread and retrograded starch. The increase in staleness and firmness of the freshly baked breads as the proportion of cross-linked starch was increased thus appears to be directly related to the extent of intermolecular bonding and crystallinity of the starch.

The relationship between the proportion of cross-linked starch and the staling and firming of bread during the 140-hour storage period cannot be assessed because of the great differences in initial freshness and firmness of breads with constant bacterial alpha-amylase content and differing proportions of cross-linked starch. Some of these breads were so stale and firm, initially, that not much change could be expected during storage.

Breads of the same starch composition were increasingly fresh and soft 2 hours from the oven, and freshness and softness were maintained to a greater degree during the 140-hour storage period as the bacterial alpha-amylase was increased (Tables I and II). Schultz *et al.* (14) found that bacterial alpha-amylase made the starch in bread more susceptible to the action of beta-amylase. This they attributed to an increase in the proportion of low-molecular starch fragments with decreased rates of retrogradation or crystallization. Recent findings of Zobel and Senti (15) show that bacterial alpha-amylase causes an increase in the crystallinity of wheat starch gels, in the crystallinity of the starch of bread, and in the rate of increase of crystallinity during aging.

The explanation of the improving effect of bacterial alpha-amylase lies in the fact that, while it increases starch crystallization, it causes other changes in starch. Miller, Johnson, and Palmer (11) observed that small amounts of bacterial alpha-amylase caused a marked increase in the quantity of soluble dextrins in fresh bread. Zobel and Senti (15) found that starch gels which contained 24 SKB units of bacterial alpha-amylase per lb. remained soft and pliable and did not crumble on aging. They attributed this effect to the breaking of bonds in the amorphous regions of the starch.

Thus, while changes in starch are shown to be of great importance in the staling and firming of bread, it can no longer be regarded that retrogradation or crystallization is the only starch property involved.

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DOUGH MOBILITY AND ABSORPTION[1]

I. HLYNKA

ABSTRACT

Farinograms by the constant-flour and constant-dough methods were obtained at varying absorptions to give a range of dough consistencies approximately 300 to 900 Brabender units and a range of absorptions (14% basis) approximately 52 to 68%. When dough mobility, obtained as the reciprocal of maximum farinograph consistency, was plotted against absorption (14% or dry flour basis), a linear relationship was established. The relationship and the data were used as a basis of calculating the absorption equivalent to an interval of 20 B.u. The factor was 0.7% absorption at the 500-unit line, but varied over the range. On the analogy with free and bound water, the amount of water that functions to confer mobility to dough, and the amount that is independent of mobility, were evaluated from the intercept of the regression equation for the absorption-mobility relation. A value of 69% water per 100 g. dry flour, independent of the property of mobility in dough, was of the same order as the values for bound water reported by Kosmin and Swanson. The basic rheological implications of the mobility-absorption relation are noted.

When dough is deformed and the deforming force removed, part of the deformation is reversible and is therefore considered to be elastic; another part of the deformation is permanent and is considered to be viscous. In a recording mixer such as the farinograph, dough is deformed by a continuous shearing force, and there is no opportunity for elastic recovery. Such instruments, therefore, measure essentially the viscous property of dough. However, to indicate that dough is not a Newtonian fluid, and that the measurement is not in precisely defined units of viscosity, the term *consistency* is used and is expressed in somewhat arbitrary Brabender units.

In basic chemistry it has been found advantageous under some conditions to use fluidity, the reciprocal of viscosity. Similarly in studies of dough, the term *mobility*, the reciprocal of consistency, has been used by Bailey and his associates (7,8), but only in a qualitative and descriptive sense. Most of the studies on the role of water in dough, however, have been in terms of consistency (6,9). This paper presents a preliminary quantitative study of the relation of dough mobility to absorption as a basic approach to the rheology of the farinograph. The concept of dough mobility implies a number of interesting consequences which will be discussed briefly.

Materials and Methods

The flour used in this study was an unbleached, improver-free,

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straight-grade sample commercially milled from a blend of Canadian hard red spring wheat. The protein content was 12.2% and ash 0.47% on a 14% moisture basis.

The farinograph used was equipped with a small stainless steel-clad mixing bowl and had a 62.9-r.p.m. drive to the bowl. The temperature of the bowl was maintained at 30.0°C. Farinograms were obtained over a range of absorption from approximately 52 to 68% (14% basis), with a corresponding range of consistencies from approximately 300 to 900 Brabender units. The same flour was used for the entire set of experiments. Both constant-flour and constant dough-weight procedures as outlined in *Cereal Laboratory Methods* (1) were used. Dough consistency was obtained in the usual way from the height of the midpoint of the curve at the maximum and was recorded in Brabender units. Dough mobility was obtained as the reciprocal of consistency, and is, of course, in reciprocal Brabender units.

Results

Relation between Mobility and Absorption, Constant-Flour Method. The first set of experiments was designed to examine the general relationship between dough mobility and absorption. A series of farinograms obtained by the constant-flour method for varying absorptions

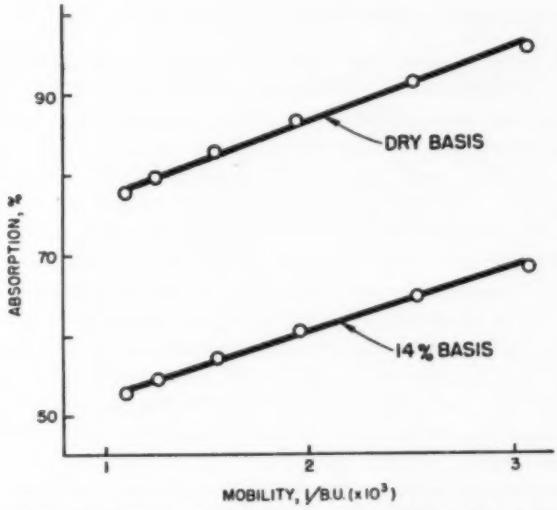

Fig. 1. Relation between dough mobility in reciprocal Brabender units and percent absorption on a 14% and dry-flour basis. The data were obtained by the constant-flour weight farinograph method.

was used to obtain the usual maximum dough consistencies ranging between 300 and 900 Brabender units. Minimum dough mobility was then evaluated as the reciprocal of consistency. Figure 1 summarizes the results. Here mobility is plotted against absorption calculated both on a 14% and on a dry-matter basis.

These results show that as the amount of water added to the flour was increased, the mobility of the dough also increased in a regular and remarkably linear manner. This linear relationship over the major part of the farinograph scale suggests that dough mobility is a more useful and perhaps a more fundamental property than dough consistency.

The regression equation for the relation between absorption, 14% moisture basis, and dough mobility for the constant-flour method is:

$$\text{Absorption (14\% basis)} = 8{,}675 \text{ mobility} + 43.45 \qquad (1)$$

The corresponding equation for absorption (dry-matter basis) as a function of mobility can be calculated from equation 1 using the relationship:

$$\text{Absorption (dry-matter basis)} = \frac{\text{absorption (14\% basis)} + 14}{0.86}$$

The resulting equation is:

$$\text{Absorption (dry-matter basis)} = 10{,}090 \text{ mobility} + 66.79 \qquad (2)$$

It will be noted in equations 1 and 2 that although both the slope and the intercept differ when a different moisture basis is used to express absorption, the basic linear relationship remains unaltered.

Relation between Mobility and Absorption, Constant-Dough Method. The constant dough-weight method for obtaining farinograms has been suggested as a more acceptable procedure than the constant-flour method. The reason for this is that the sensing element (i.e. mixing blades) engages the same weight of dough at any absorption. In the constant flour-weight method the weight of the dough engaged by the mixing blades presumably increases with increasing absorption. Accordingly, in the next set of experiments a series of farinograms was obtained by the constant dough-weight method for varying absorptions. The data are again plotted as mobility against absorption and are shown in Fig. 2. Absorption was calculated on a 14% moisture basis as well as on a dry-matter basis.

The regression equation for the relation between absorption, 14% basis, and dough mobility, constant-dough method is:

$$\text{Absorption (14\% basis)} = 7{,}585 \text{ mobility} + 45.18 \qquad (4)$$

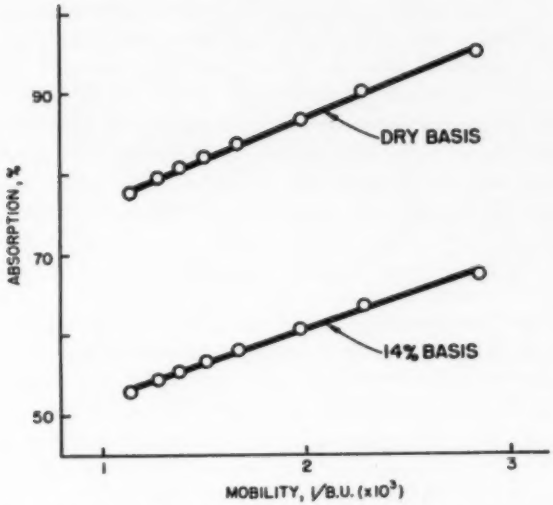

Fig. 2. Relation between dough mobility in reciprocal Brabender units and percent absorption on a 14% and dry-flour basis. The data were obtained by the constant dough-weight farinograph method.

The corresponding equation with absorption calculated on a dry-matter basis is:

$$\text{Absorption (dry-matter basis)} = 8{,}820 \text{ mobility} + 68.81 \qquad (5)$$

It is seen once again that a linear relationship between absorption and mobility is maintained for the constant-dough method. In other words, the effect of the increasing dough weight with absorption in the constant-flour method is systematic. A close comparison of the results obtained by the two methods shows that the differences between them are quite small.

Linear absorption-mobility plots were also obtained for a number of other flours, indicating that this relationship is general.

Change in Absorption Corresponding to a Change of 20 Brabender Units in Dough Consistency. Cereal Laboratory Methods (1) states that 20 Brabender units in dough consistency corresponds to approximately 0.8% in absorption. This factor is extremely useful in the laboratory in estimating the adjustment in absorption that is necessary when the farinogram obtained in a preliminary trial (constant-flour method) is not centered exactly about the 500 B.u. line. The linear relationship between mobility and absorption described in this paper offers a precise method of evaluating this factor.

Table I presents data on the calculation of absorption equivalent to 20 Brabender units' consistency for various ranges. The first column shows the individual intervals, in B.u., that were selected. The reciprocals of these values, i.e. mobilities, are given in the second column. These values in turn were used in the regression equation 1 for the constant-flour method, 14% moisture basis, to calculate the absorption (column 3) corresponding to each mobility. Finally, the mobilities, absorption, and the regression coefficient of equation 1 were used in the equation

$$y_2 - y_1 = m \ (x_2 - x_1) \tag{6}$$

to calculate the change in absorption $(y_2 - y_1)$ corresponding to an interval of 20 Brabender units expressed as mobilities $(x_2 - x_1)$. The last column gives the absorption that corresponds to successive 20 Brabender unit intervals of consistency from 400 to 600 units.

The above data show that at 500 Brabender units for the flour, machine, and conditions used, a change of 20 B.u. corresponds to 0.7% absorption. They further show that this factor is not constant, but is increasingly higher as the deviation below 500 B.u. increases, and lower as the deviation above 500 B.u. increases. The significant feature, how-

TABLE I

CHANGE IN ABSORPTION CORRESPONDING TO A CHANGE OF 20 BRABENDER UNITS IN DOUGH CONSISTENCY FOR DIFFERENT RANGES

CONSISTENCY	MOBILITY	ABSORPTION (14% MOISTURE BASIS)	CHANGE IN ABSORPTION PER 20 B.U. IN THE RANGE
B.u.	$1/B.u. \times 10^3$	%	%
400	2.500	65.14	
			1.03
420	2.381	64.11	
			0.94
440	2.273	63.17	
			0.86
460	2.174	62.31	
			0.79
480	2.083	61.52	
			0.72
500	2.000	60.80	
			0.67
520	1.923	60.13	
			0.61
540	1.852	59.52	
			0.58
560	1.786	58.94	
			0.53
580	1.724	58.41	
			0.50
600	1.667	57.91	

ever, is that a method is now available for precisely determining the equivalence between consistency and absorption.

Discussion

In reflecting upon the fundamental aspects of the findings presented in this paper, it seems entirely logical that water content should be simply and directly related to dough mobility. This implies that one of the main functions of water in dough is merely to confer the property of mobility to the dough. In addition it is well known, of course, that some of the water functions in other ways, as for example in simple absorption by the starch granules and in the hydration of gluten. We thus have a picture very similar to that of "free" and "bound" water. In fact, one can evaluate the amount of water that may be considered to function solely to confer mobility to dough and make an interesting comparison with the values for "free" and "bound" water in dough as reported in the literature.

To evaluate the amount of water in dough that is associated with mobility, and the water that is independent of mobility, it is necessary to examine the significance of the regression equation 5, and Fig. 2, which relate water absorption on a dry-matter basis with mobility as determined by the constant-dough method.

$$\text{Absorption (dry-matter basis)} = 8{,}820 \text{ mobility} + 68.81$$

According to this equation, as the total water is decreased, the water associated with dough mobility (analogous to free water) will eventually decrease to zero. At this point only the water that is independent of mobility (analogous to bound water) is present. This value is directly given by the intercept of the regression equation and is 69% of moisture-free flour.

It is now of interest to examine the data reported in the literature for free and bound water in dough. Kosmin (4) reported that 80% of the water added was bound water. Recalculating from the range given, one obtains a range of 51 to 66%, and from mean data 58% bound water per 100 g. dry flour. Swanson (12) stated that two-thirds of the total water in dough was in the bound form. When recalculated on the basis of 100 g. dry flour his result is identical with Kosmin's. Considering the wide differences possible in flour characteristics, the data of Kosmin and Swanson may be considered as generally consistent with our data derived from dough mobility measurements.

However, some data on bound water (on dry-flour basis) are significantly lower. Newton and Cook (10) reported 29 to 52%; Skovholt and Bailey (11) 42 to 45%; Kuhlmann and Golossowa (5) 44 to 48%; Vail

and Bailey (13) 29%; and finally Baker, Parker, and Mize (2) reported 27% as bound water on a dry-matter basis. Some of these differences are no doubt due to differences in methods used for estimation of bound water and the assumptions that were implicit in the methods. Nevertheless the analogy between water in dough that is independent of mobility and "bound water" is interesting.

Another interesting aspect of considering water in dough as associated with mobility and with absorption is that it should be possible to detect experimentally changes in equilibrium from one form of water to another. Consider, for instance, the hydration of flour in the initial formation of dough. Hydration would require a decrease in the amount of water associated with mobility, and dough consistency should rise. The experience, however, is that if the farinogram is interrupted to presumably allow for hydration, the height of the curve generally decreases. This indicates that hydration of flour under usual conditions is rapid.

One final aspect of the present study may be noted. The concept of dough mobility makes obvious a common rheological basis in the farinograph method of determining absorption and in the Halton (3) dough-extrusion method. Both the dough consistency in the farinograph and the extrusion time in the "research water absorption meter" can be advantageously viewed in terms of dough mobility.

This paper has presented the preliminary and more obvious aspects of dough mobility in relation to absorption. Further work aimed at understanding some of the fundamental rheological principles underlying the farinograph and applying them to the study of dough are in progress.

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EXPANSION OF NEW AND OLD RICE DURING COOKING[1]

H. S. R. DESIKACHAR AND V. SUBRAHMANYAN

ABSTRACT

The increase in length and breadth of rice during cooking was used to measure its swelling quality. Old rice could increase in length much more than new rice, and without disintegration. The terminal portions along the long axis and the ventral segment along the short axis of the grain exhibited greater expansion than the corresponding central portions and the dorsal segment along the long and short axes, respectively, of the grain. Fragility of the cell walls in new rice was evidenced by their tendency to burst earlier during cooking than did those of old rice. The cell walls in parboiled rice exhibited an intact structure even after cooking for 30 minutes.

The tendency of fresh rice to cook to a pasty mass enclosing the viscous, sticky gruel between particles of cooked rice has been mentioned previously (1,2,6). This property of new rice often leads to errors in the evaluation of its swelling property. In an effort to minimize such errors, the possibility suggested itself of cooking individual grains (a) separately and (b) in an excess of water for the purpose, respectively, of preventing the sticking together of grains and diluting the gruel. Encouraging results were obtained in preliminary experiments. A more detailed study of the problem was therefore undertaken to determine the progressive swelling of fresh and old rice during the cooking process.

The increase in weight and volume was first determined as a measure of swelling, but this was found time-consuming. Measurement of increase in length and breadth as a result of cooking was quicker and

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easier in manipulation and gave consistent results, and was therefore adopted in all subsequent work.

Cooking consists essentially of the swelling of the cell contents, especially of starch granules. In fresh rice, the cell contents escape into the cooking water by rupture of cell walls. For this reason, a qualitative difference in cell-wall structure between new and old rice after cooking is to be expected. Sections of new and old rice were therefore examined under the microscope to find out whether any such difference did exist in the cell-wall structure after cooking.

Materials and Methods

Milled rice, obtained from paddy 1 month old (Co 11 variety), secured from a paddy breeding station, served as the source of "new" rice in this study. Paddy in the same variety from a previous year's harvest and grown on the same farm under similar cultural conditions served as the source of "old" rice.

The grains were measured for initial length and breadth. They were then put in separate test tubes containing 2 ml. water and cooked in a boiling-water bath for different periods. The excess water was drained off and the dimensions of the cooked grains were again determined. As it was found from the above experiments that old rice increased in length much more than new rice, the possibility of differential expansions in segments of new and old rice along the length and breadth of the grains was investigated. The grain was divided into segments by thin points marked with a concentrated solution of Crystal Violet. Four segments of approximately equal length along the long axis were marked out and the expansion of each segment was measured. The expansions of the two end segments and those of the two middle segments were pooled separately for calculating the average expansion ratio of terminal and central segments respectively in each grain. Similarly the expansion of the top and bottom (ventral and dorsal) seg-

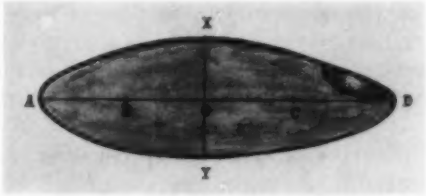

Fig. 1. Section of rice grain explaining the segments whose expansions were measured. Along long axis AD: AB, CD, terminal segments; BO, OC, central segments. Along short axis XY: OX, top segment; OY, bottom segment. AXD, top or ventral line of fusion.

ments along the short axis was also measured (see Fig. 1 for explanation).

Measurements were made by means of a calibrated micrometer fitted into the eyepiece of a microscope. Observations were made at low magnification (about 20×), the top surface of the grain being focused for observation. The indicator used for marking had spread to some extent after cooking, and hence measurements of length were made from center to center of the markings both before and after cooking. To allow for variation in initial lengths of grain, expansions were expressed as a ratio of final to initial length.

For microscopic examination freehand sections were used throughout this study. Raw grains had to be softened previously by soaking in water for 24 hours; cooked grains were soft enough for direct sectioning. Hematoxylin or safranin was used for staining. Sections were examined mainly with a view to observing the integrity or intactness of cell walls both before and after cooking.

Results and Discussion

Preliminary observations were first made to determine variations in expansion from grain to grain and to determine the minimum number of grains that had to be used for getting average values. Means of seven determinations of expansion ratios were 1.34 and 1.53 for new and old rice respectively, and their respective standard deviations were 0.10 and 0.07. The differences in expansion of new and old rice were statistically significant at the 1% level. In subsequent experiments averages of six grains were taken in computing expansions. In certain cases where the grain either broke or disintegrated during the later stages of cooking, the average of four grains has been taken.

TABLE I

EXPANSION RATIO[a] OF NEW AND OLD RICE DURING COOKING

COOKING TIME	LENGTH		BREADTH	
	New	Old	New	Old
minutes				
2	1.06	1.09	1.10	1.10
3	1.11	1.18	1.16	1.18
4	1.21	1.26	1.31	1.26
5	1.25	1.30	1.37	1.32
6	1.38	1.47	1.38	1.31
8	1.41	1.58	1.43	1.39
10	1.42	1.62	1.43	1.49
12	1.43	1.65	1.49	1.46
15	1.41	1.64	1.46	1.57

[a] The expansion of grains is expressed as the ratio of cooked to uncooked length of grain.

Table I presents data for the rate of expansion in length and breadth of new and old rice during cooking. Old rice had the tendency to expand more along its length than new rice at all stages of cooking. The pattern of increase in breadth was, however, less regular; the expansion of new rice was sometimes more than that of old rice. One possible reason for this irregularity is the difficulty involved in measuring breadth; the grain cannot be made to rest on the same plane both before and after cooking. Another reason is splitting of the grain along the line of fusion on the ventral side (Fig. 2).

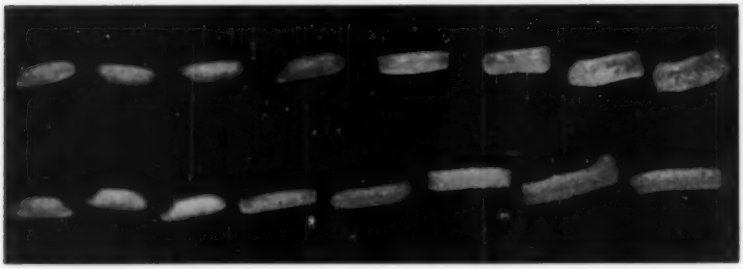

Fig. 2. Comparative changes in size and shape of new (top) and old (bottom) rice during cooking.

A notable feature of these observations, however, is that old rice can be cooked safely up to 15 minutes until the center of the grain is cooked to a soft consistency. On the other hand, if new rice is cooked beyond 10 minutes to a stage when the center becomes soft, a large number of grains burst along the ventral line of fusion, dispersing the cell contents to the cooking water. This line, therefore, becomes flattened and the grain has a hollow appearance when viewed from the top (see Fig. 2).

The average expansion of different segments along the length and breadth of the grain is presented in Tables II and III, respectively.

TABLE II

DIFFERENTIAL EXPANSION RATIO[a] OF SEGMENTS ALONG THE LENGTH OF RICE GRAIN DURING COOKING

Boiling Time	New		Old	
	Terminal	Central	Terminal	Central
minutes				
6	1.31	1.23	1.37	1.26
10	1.32	1.25	1.50	1.49
15	1.37	1.29	1.51	1.48

[a] The expansion of grains is expressed as the ratio of cooked to uncooked length of grain.

TABLE III

DIFFERENTIAL EXPANSION RATIO[a] OF TOP AND BOTTOM SEGMENTS ALONG THE BREADTH OF RICE AFTER 10 MINUTES' COOKING

	NEW	OLD
Bottom	1.41	1.27
Top	1.71	1.61

[a] The expansion of grains is expressed as the ratio of cooked to uncooked length of grain.

Both in the new and the old rice samples the terminal portions expanded in length more than the central portions, particularly in the initial stages of cooking. This is probably because the central portions have to expand against a longer column of length than the terminal portions, which are free to expand. Alternatively, the end segments have a larger area of exposed surface relative to the central segments and might therefore absorb more water and elongate more. As to the expansion along the short axis, there are definite indications that the top portion (toward the germ side) suffered greater expansion than the bottom portion. The reason for this is not very clear. It may be that the cells are more free to expand toward the ventral line of fusion (suture), which is a weak line morphologically. During puffing, the rice grain bursts along this line of fusion. Also, the thickness of the aleurone layer is smaller along the ventral side than along the dorsal side of the grain (5).

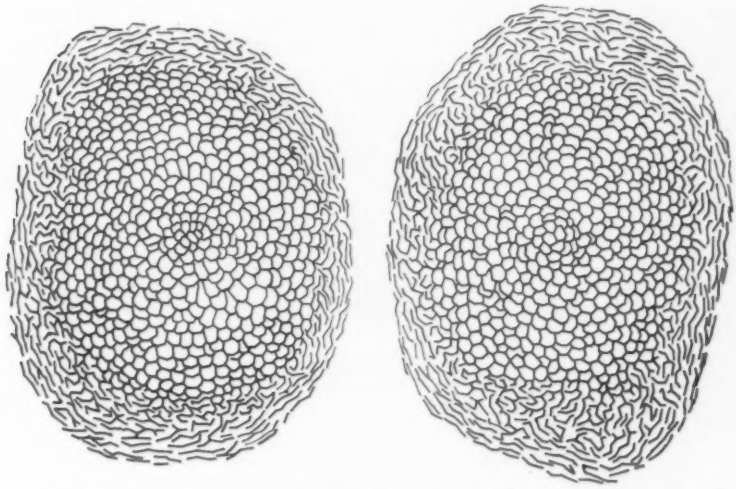

Fig. 3. Transverse section of new and old rice cooked for 12 minutes showing diagrammatically the outline of cells.

When serial transverse sections are examined, the bundle of cells parallel to the short axis is often different in structure from the other cells in the grain (compare: "Wheat flour," 3,4). These cells are long and tubular or prismatic, whereas the majority of the other cells are bigger and present the usual hexagonal shape (see Fig. 5, right). Whether this characteristic distribution of prismatic cells is responsible for the differential expansion along the top and bottom halves

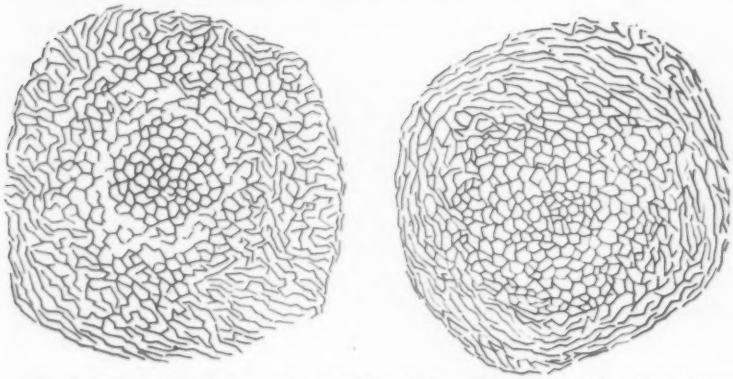

Fig. 4. Transverse section of new and old rice grains cooked for 20 minutes showing diagrammatically the relative abundance of disrupted cells.

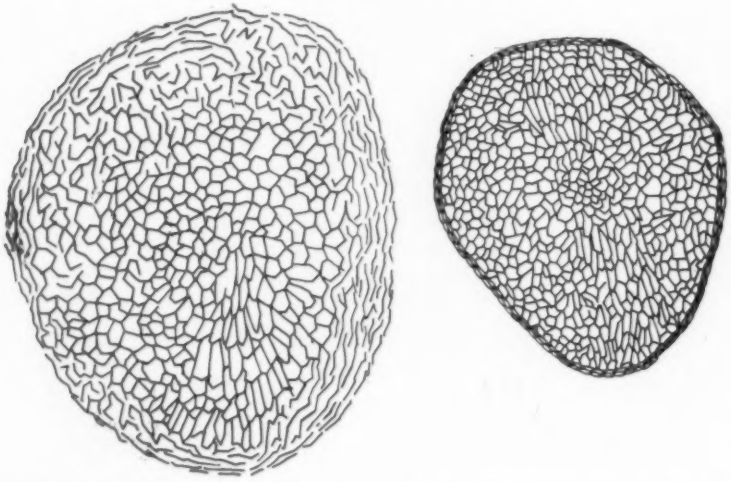

Fig. 5. Left: Diagrammatic representation of the cell pattern in parboiled rice cooked for 30 minutes. Right: Transverse section of rice showing, diagrammatically, endosperm cells of different shape.

of the short axis of the grain is to be further investigated. The causes for greater expansion of old rice, particularly along the long axis, also need further investigation.

A diagrammatic representation of the pattern of cell structure in new and old rice when cooked for 12 and 20 minutes is given in Figs. 3 and 4 respectively. Figure 5, left, gives the structure of parboiled rice cooked for 30 minutes; Fig. 5, right, represents the distribution of different types of endosperm cells referred to above. From Figs. 3 and 4 it is clear that when old and new rice were heated for the same period, a larger number of cells in new rice had their cell walls broken, whereas in old rice the cells retained to a larger extent the integrity of cell structure. In parboiled rice, even after 30 minutes of cooking, the cells preserved their original shape and remained structurally intact. These studies have therefore revealed that, owing to fragility or lack of sufficient strength of the cell walls, the cells of new rice are more likely to give way to osmotic pressure developed inside the cells. Hardening of the grain during parboiling either hardens the cell walls or imparts to them a resilient property so that they can resist the high pressure developed inside. These data, therefore, indicate that besides the change noted earlier in the properties of the starch and protein (1), the component of the cell wall also undergoes some changes that harden it. The exact nature of these changes needs to be further elucidated. A more detailed investigation of the cellular structure of the new and old rice grain using thin microtome sections is also necessary to confirm and extend the present findings.

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March, 1959

Page 169, L. T. KISSELL:
In line 8, for *daily whey* read *dairy whey*.

EDITORIAL POLICY

Cereal Chemistry publishes scientific papers dealing with raw materials, processes, or products of the cereal industries, or with analytical procedures, technological tests, of fundamental research, related thereto. Papers must be based on original investigations, not previously described elsewhere, which make a definite contribution to existing knowledge.

Cereal Chemistry gives preference to suitable papers presented at the Annual Meeting of the American Association of Cereal Chemists, or submitted directly by members of the Association. When space permits, papers are accepted from other scientists throughout the world.

The papers must be written in English and must be clear, concise, and styled for Cereal Chemistry.

Manuscripts for publication, and correspondence directly pertaining to them, should be sent to the Editor-in-Chief, CEREAL CHEMISTRY, 1955 University Ave., St. Paul 4, Minn. All other correspondence, including circulation, advertising, or other business, should be directed to the Managing Editor at the above address.

Manuscripts of published papers will be kept on file for one year. After that time they will be destroyed unless other instructions have been received from the author. Original graphs, etc., and negatives of all illustrations are returned to the author immediately upon publication.

SUGGESTIONS TO AUTHORS

General. Authors will find the last volume of *Cereal Chemistry* a useful guide to acceptable arrangements and styling of papers. "On Writing Scientific Papers for Cereal Chemistry" (*Trans. Am. Assoc. Cereal Chem.* 6:1–22. 1948) amplifies the following notes.

Authors should submit two copies of the manuscript, typed double spaced with wide margins on 8½ by 11 inch white paper, and all original drawings or photographs for figures. If possible, one set of photographs of figures should also be submitted. Originals can then be held to prevent damage, and the photographs can be sent to reviewers.

Editorial Style. A.A.C.C. publications are edited in accordance with *A Manual of Style,* University of Chicago Press, and *Webster's Dictionary.* A few points which authors often treat wrongly are listed below:

Use names, not formulas, for text references to chemical compounds. Use plural verbs with quantities (6.9 g. were). Figures are used before unit abbreviations (3 ml.), and % rather than "per cent" is used following figures. All units are abbreviated and followed by periods, except units of time, which are spelled out. Repeat the degree sign (5°–10°C.). Place 0 before the decimal point for correlation coefficients ($r = 0.95$). Use * to mark statistics that exceed the 5% level and ** for those that exceed the 1% level; footnotes explaining this convention are no longer required. Type fractions on one line if possible, e.g., $A/(B + C)$. Use lower case for farinograph, mixogram, etc., unless used with a proper name, i.e., Brabender Farinograph. When in doubt about a point that occurs frequently, consult the Style Manual or the Dictionary.

For more detailed information on manuscript preparation see
May 1959 issue (Cereal Chem. 36: 318–319)

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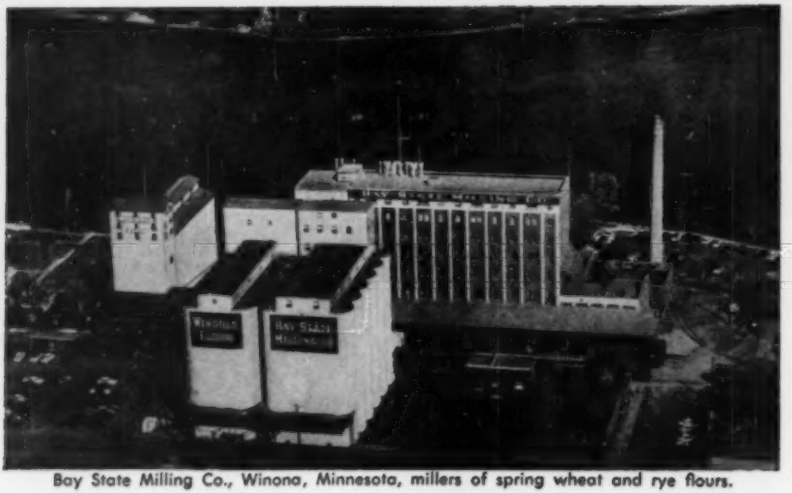

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